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ADB155185

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DEPARTMENT OF THE ARMY

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FOR THE COMMANDER

CORNELIUS R. FAY III
Lieutenant Colonel, MS
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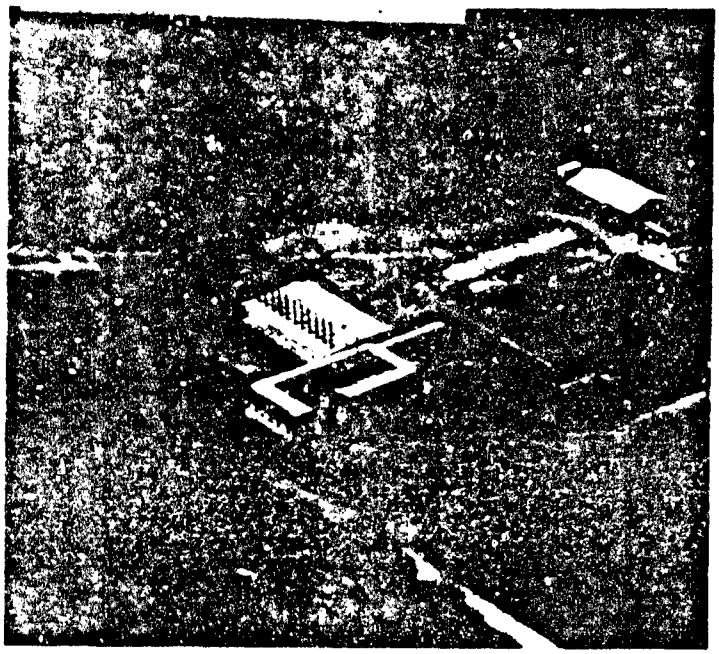
REPORT



FINAL REPORT

Task 89-06: Determination of the Bioequivalence of HI-6 and Atropine When Delivered by Wet/Dry Autoinjector or Syringe in Sheep: A Pharmacokinetic and Efficacy Evaluation

To

U.S. Army Medical Research and Development Command

Institute of Chemical Defense

Battelle
Putting Technology To Work

April, 1991

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Distribution authorized to U.S. Government agencies and their contractors; Reason - Critical Technology; April 30, 1991.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Battelle Columbus Operations; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION: U.S. Army Medical Research Institute of Chemical Defense ATTN: SGRD-RMI-S
6c. ADDRESS (City, State, and ZIP Code): 505 King Avenue, Columbus, OH 43201-2693	7b. ADDRESS (City, State, and ZIP Code): Aberdeen Proving Ground, MD 21010-5425
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research and Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Contract No. DAMD17-89-C-9050
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21702-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 63002A; PROJECT NO. 3M 2630020 995; TASK NO. AI; WORK UNIT ACCESSION NO. DA 317949

11. TITLE (Include Security Classification)
A Medical Research and Evaluation Facility (MREF) and Studies Supporting the Medical Chemical Defense Program

12. PERSONAL AUTHOR(S) Olson, Carl T., Dill, Garrett S., Menton, Ronald G., Kiser, Robyn C., Blank, James A., Snider, Thomas A., Matthews, M. Claire., Hayes, Timothy L., Miller, Pers'ng

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 891001 TO 910415	1991 April	229

16. SUPPLEMENTARY NOTATION
Task 89-06: Determination of the Bioequivalence of HI-6 and Atropine When Delivered By Wet/Dry Autoinjector or Syringe in Sheep: A Pharmacokinetic and Efficacy Evaluation

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	15	
06	20	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Autoinjector, Sheep, Pharmacokinetics, Efficacy, CSM, Soman (GD), Atropine, Pralidoxime Chloride (2-PAM), Mark I Autoinjector, HI-6, Wet-Dry Autoinjector

19. ABSTRACT (Continue on reverse if necessary and identify by block number)
A study was instituted in sheep to compare efficacy against Soman and the pharmacokinetics of atropine and HI-6 by wet/dry autoinjector and by syringe. The efficacy in sheep against Soman of Atropine/HI-6 delivered by wet/dry autoinjector was also compared to the efficacy of atropine/pralidoxime chloride when delivered by syringe. The "aging" rate of GD was also determined in sheep erythrocytes.

There were no statistical differences in the LD_{50}'s of sheep treated with atropine/HI-6 using syringes or wet/dry autoinjectors. The 48-hr GD LD_{50} estimated for sheep treated with atropine/2-PAM was not significantly less than the LD_{50} of sheep treated with atropine/HI-6 using syringes or wet/dry autoinjectors. A PR was determined for atropine/HI-6 treatment.

Based on analyses of HI-6 and atropine pharmacokinetic parameters there were no statistically significant differences in the two injection techniques. The aging rate of sheep RBC's is highly dependent upon the GD-AChE incubation temperature.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT: ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION: Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mary Frances Bostian	22b. TELEPHONE (Include Area Code): (301) 663-7325; 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

FINAL REPORT

Contract DAMD17-89-C-9050
A Medical Research and Evaluation Facility (MREF) and Studies
Supporting the Medical Chemical Defense Program

on

TASK 89-06: DETERMINATION OF THE BIOEQUIVALENCE OF HI-6 AND ATROPINE WHEN DELIVERED BY WET/DRY AUTOINJECTOR OR SYRINGE IN SHEEP: A PHARMACOKINETIC AND EFFICACY EVALUATION

to

U.S. ARMY MEDICAL RESEARCH
AND DEVELOPMENT COMMAND

April, 1991

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In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health (NIH), Publication No. 86-23, Revised 1985).

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FINAL REPORT

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TASK 89-06:

DETERMINATION OF THE BIOEQUIVALENCE OF HI-6 AND ATROPINE WHEN DELIVERED BY WET/DRY AUTOINJECTOR OR SYRINGE IN SHEEP: A PHARMACOKINETIC AND EFFICACY EVALUATION

to

U.S. ARMY MEDICAL RESEARCH
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April, 1991

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TABLE OF CONTENTS

TABLE OF CONTENTS
(Continued)

LIST OF TABLES

LIST OF TABLES
(Continued)

LIST OF FIGURES

TASK 89-06: DETERMINATION OF THE BIOEQUIVALENCE OF HI-6 AND ATROPINE WHEN DELIVERED BY WET/DRY AUTOINJECTOR OR SYRINGE IN SHEEP: A PHARMACOKINETIC AND EFFICACY EVALUATION

1.0 INTRODUCTION

The oxime 1-2-hydroxyiminomethyl-1-pyridino-3-(4-carbamoyl-1-pyridino)-2-oxapropane dichloride (HI-6) given in conjunction with atropine appears to be the most effective available treatment of pinacolyl methylphosphonofluoridate (soman; GD) intoxication. The efficacy of HI-6 in countering the effects of GD has been repeatedly demonstrated in a variety of animals, including rodents, rabbits, dogs, and monkeys.[1,2,3,4] A major obstacle to the development of HI-6 for military use is its marked instability in solution. Current U.S. Army autoinjector systems for treating nerve agent intoxication package all components in solution. An effort is underway to develop a delivery device, a wet/dry autoinjector, which will allow packaging of HI-6 in its stable, crystalline form. In such a system, the HI-6 would be rapidly dissolved in an atropine solution so that within a matter of seconds the mixture of atropine and HI-6 could be injected. Since adequate shelf life (more than 3 years) can be expected for HI-6 while in a dry form, the development of a wet/dry autoinjector appears to be critical to the successful fielding of this oxime as a nerve agent antidote.

No *in vivo* studies have been accomplished to validate the wet/dry autoinjector as an effective method of introducing an emergency antidote for nerve agent intoxication. Substantial differences may exist between the experimental administration of previously prepared solutions of atropine and HI-6 given by syringe and the proposed administration of the compounds using a wet/dry autoinjector. Potential problems with a wet/dry autoinjector delivery system include:

Any delay in administration of nerve agent antidote because of time required to form an HI-6 solution may result in therapeutic failure. Time to treatment becomes critical once signs of nerve agent intoxication are manifested.

The dispersion pattern of the admixed solution in tissue may alter the absorption of the antidote compounds. The design of the

autoinjector is such that the tissue dispersion pattern of injected material cannot be simulated in the laboratory with the use of syringes.

A relatively small deliverable dose of HI-6, due to the small volume capacity of the autoinjector, may not be adequate. Although the data base for HI-6 suggests efficacy in treating GD intoxication, virtually all studies have been done in small animals (less than 10 kg) at relatively large doses (20-200 mg/kg). As packaged in available autoinjectors, the dose of HI-6 to a standard 70 kg man ranges from 5.7 to 7.1 mg/kg per injection. It is not certain that HI-6 can provide improved survivability or reduced morbidity relative to the currently fielded nerve agent antidote regimen if its use is constrained by formulation and/or doctrinal considerations.

This task was initiated at Battelle's Medical Research and Evaluation Facility (MREF) to:

1. Compare the efficacy of atropine and HI-6 delivered intramuscularly (IM) by wet/dry autoinjector with their efficacy when delivered IM by conventional syringe in the treatment of GD intoxication in sheep.

2. Compare the efficacy of atropine/HI-6 delivered by wet/dry autoinjector to the efficacy of atropine/pralidoxime chloride (2-PAM) delivered by the Mark I (MKI) autoinjector system in preventing the effects of GD intoxication in sheep.

3. Determine and compare pharmacokinetic parameters in sheep for atropine and HI-6 when injected IM by syringe and when delivered by wet/dry autoinjector.

4. Determine the "aging" rate of GD in sheep erythrocytes.

2.0 EXPERIMENTAL DESIGN

2.1 Test Animals

Sheep were used for this study because of their known response to organophosphonate (OP) chemical surety materiel (CSM), because of similarities with man in body weight, and because sheep have been used in similar pharmacokinetic studies with IM administered drugs.[5,6,7,8] Approximately 1-year-old wethers of Rambouillet-Columbia breeding were obtained from Thomas D. Morris, Inc., Reisterstown, MD. All sheep had serology performed prior to shipment to the MREF and were negative for antibody titers for the Q fever causative organism, Coxiella burnetii. Upon arrival at the MREF, sheep were examined by a veterinarian and blood and fecal samples were obtained for clinical pathological and gastrointestinal parasite evaluations. Sheep were held in quarantine for a minimum of 7 days prior to use in a study. All animals were tagged in the ear to retain positive identification and were maintained in an outdoor fenced area with available shelter until brought into the laboratory for experimentation. Sheep were fed Purina Rumilab® with limited quantities of locally purchased hay. Water was supplied from Battelle's West Jefferson water system ad libitum. The water is analyzed quarterly for potability and annually for contaminants. No contaminants which would interfere with the results of the study are known to be present in the water or feed.

Sheep were shorn, brought into the laboratory, and maintained on straw bedding in animal rooms kept at 65 ± 15 F with a relative humidity of 50 ± 20 percent. Fluorescent lighting with a light/dark cycle of 12 hr each per day was used. Sheep were acclimated to a sling suspended from a stand for a minimum of 20 min per day for 2 days prior to experimentation, and they routinely adapted rapidly to this method of restraint. Each animal was weighed using a Nordic Forge scale (NASCO, Fort Atkinson, WI) and animals were homogenized by weight into groups for efficacy testing of treatments for GD intoxication. Sheep weighed between 60 and 100 kg at the time of testing and appeared to be in good physical condition.

2.2 Materials and Methods

2.2.1 Chemistry

Atropine and 2-PAM contained in MKI antidote injection systems and atropine and HI-6 contained in wet/dry antidote injection systems as well as additional HI-6 in a solid state were provided by the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD). Sufficient numbers of antidote injection systems from the same lot were provided so that efficacy and pharmacokinetic studies could be performed. Atropine sulfate monohydrate (Aldrich, Lot No. EW01602HU, 98.6 percent pure) was purchased and a solution prepared in the same formulation as the atropine solution used in the wet/dry autoinjectors (proprietary information). This atropine solution was used as diluent for the HI-6 in preparing admixed atropine/HI-6 solutions for dosing of sheep by syringe on each day of efficacy testing. A sample of the atropine/HI-6 solution prepared on each day of dosing was analyzed to confirm desired concentrations. Commercially available atropine sulfate injectable solution (Atropine L.A., Butler, Columbus, OH) was purchased locally for use as supplemental atropine in efficacy studies.

The MKI system consists of two separate injectors, one for atropine (Atropen) and one for 2-PAM. Removing either tubular plastic "pen" from the common holder arms a spring-loaded mechanism which forces a hypodermic needle from the pen when its head is pressed against the skin. The same spring mechanism advances a plunger which causes injection of the contents of the pen. The wet/dry autoinjector system (ASTRA Autoinjektor) delivers atropine and HI-6 in a single injection. The top portion of the injector is rotated to break a seal and the injector is shaken for ten sec to mix the atropine solution and powdered HI-6. A safety ring is removed, the injector held against the skin, and the red top is pressed to initiate injection of the contents. Both autoinjector systems are kept in place against the skin for 10 sec after activation to ensure complete injection.

GD was supplied by USAMRICD. Purity of GD stored at Battelle is periodically confirmed by Battelle chemists. For dosing of sheep, a nominal 1.7 mg of GD per mL of physiologic saline solution was prepared for this

study, and aliquots were stored in amber 10-mL serum vials at approximately -70 C. After each dosing day, samples of dosing aliquots were analyzed by gas chromatography.

2.2.2 GD LD_{50} Estimation

A 48-hr GD LD_{50} was estimated for this population of sheep. Sheep were given IM injections of GD in the caudolateral area of the right thigh in the region of the semitendinosus muscle. Doses were selected by the study statistician using an up-and-down design in which only one or two sheep were dosed a day. Twenty-three gauge, one-inch needles were used for all IM injections. To obtain maximum accuracy in delivered doses, syringes used for dosing GD were Hamilton (Reno, NV) microliter syringes of the smallest compatible volume. Syringes were filled to no more than 95 percent of labeled volume. Individual, labeled syringes were loaded with the calculated volumes of GD prior to the start of dosing, weighed, and placed on ice until used. After dosing was completed, syringes were weighed again to determine weight losses. Weight losses were used to calculate the volumes actually delivered. At the time of injection of these exempt concentrations of GD, sheep were restrained in a sling and positioned at the face of a hood approved for the use of highly hazardous chemicals. Experimentation performed under Task 88-38 of a prior contract had established the 48-hr GD LD_{50} in sheep at 6.6 µg/kg body weight. Using a common slope model (i.e., using the slope determined for the GD dose-lethal response curve determined in Task 88-38), an LD_{50} was estimated by a probit model. After each day of testing, a statistical hypothesis test was conducted to determine if the LD_{50} estimated for the current population of sheep was similar to the 6.6 µg/kg estimated in Task 88-38. The study was designed to use no more than 10 sheep in estimating an LD_{50}. If, after five or more sheep were dosed, the estimated LD_{50} was within the 95 percent confidence limits (5.1-7.1 µg/kg) of the LD_{50} estimated in Task 89-38, the LD_{50} estimated in Task 88-38 would be accepted as the LD_{50} for sheep in this study and no further sheep would be injected for the purpose of establishing an LD_{50} in untreated animals.

2.2.3 Treatment Efficacy Evaluation

The second phase of this study was designed to compare the GD intoxication treatment efficacies of three treatment regimens: 1) atropine/HI-6 given by three wet/dry autoinjectors, 2) equivalent doses of admixed atropine and HI-6 given via three hypodermic syringes, and 3) atropine/2-PAM given by three MKI autoinjector systems. To ensure that efficacy of reactivator therapy was not adversely affected by an insufficient dosage of anticholinergic, supplemental atropine was administered IM to provide a total atropine sulfate equivalent dose of approximately 0.5 mg/kg, as recommended for treatment of OP intoxication in sheep.[9,10] GD was given IM in the caudolateral muscles of the right thigh and one min later all treatments were given IM simultaneously in the anterior lateral area of the left thigh in the region of the vastus lateralis head of the quadriceps femoris muscle with a one inch or more separation of injection sites. Autoinjectors were weighed prior to use, and again, after injections were made, to confirm delivery of autoinjector contents.

Experiments were conducted in a stagewise fashion, using only a few sheep in each treatment group per day, to determine the LD_{50} for each group. The LD_{50}s and slopes of the dose-response curves were compared after each stage to determine any significant differences in efficacies of therapy. The maximum number of sheep to be used in each of the atropine/HI-6 via syringe and atropine/2-PAM (MKI) treatment groups was set at 25, and the maximum number of sheep in the atropine/HI-6 via wet/dry autoinjector group was set at 35. If significant differences ($P \leq 0.05$) in treatment efficacies were determined with fewer sheep, experimentation would cease at that time. If, after a minimum of 10 sheep in each of the three groups had been challenged and treated, there was not a statistical difference in LD_{50} values between atropine/2-PAM and atropine/HI-6 treated sheep, the USAMRICD Task Area Manager and Contracting Officer's Representative were to be consulted and a course of further action planned.

Sheep were returned to an animal holding room following GD and treatment injections, removed from slings, and observed for 48 hr for signs of GD intoxication, including muscle fasciculations, tremors, convulsions,

excessive salivation/bronchial secretions, and prostration. Sheep were closely and continuously observed for the first two hr following GD injection and then at decreasing frequency for the remainder of the 48 hr period. Any signs observed and their times of onset were recorded when feasible. The incidence and recovery from sublethal responses such as convulsions or prostration were statistically analyzed, as feasible, to determine any differences in incidence or severity of effects in sheep receiving different therapies. Animals dying on study were submitted for necropsies and all gross lesions were recorded. No tissue sample collection was performed. After necropsy, remains were incinerated. Sheep still alive at the end of the 48-hr observation period were euthanatized and cremated.

2.2.4 Pharmacokinetic Studies

In pharmacokinetic studies, sheep were given equivalent doses of atropine/HI-6 either with three wet/dry autoinjectors or with three conventional syringes. Sheep were given IM injections in the anterior lateral area of the right thigh in the area of the vastus lateralis head of the quadriceps femoris muscle. Solutions delivered by hypodermic syringes were prepared in the same formulation as that used in autoinjectors (autoinjector data provided by USAMRICD). After a minimum one-week washout period, the same sheep were injected again using the injection technique not used originally. The study was designed so that an equal number of sheep would be given atropine/HI-S by each of the injection techniques on each day of testing with a total of eight sheep treated with both techniques.

Ten-mL blood samples were taken from a jugular vein through an indwelling catheter (French 8 Catheter Sheath Introducer System, Cordis Corp., Miami, FL) or with a disposable 10-mL syringe and 18 gauge, 1.5 inch needle (Becton Dickinson, Rutherford, NJ) if the catheter was not patent. The 17-cm rigid plastic vessel dilator, rather than the sheath assembly itself, was used as the catheter because the flexible plastic sheath assembly would collapse and become crimped whenever an animal turned his head to the side. The vessel dilator was loosely fixed in place with a stay suture placed in the skin, and a three-way stopcock was attached to the catheter.

Blood samples were taken a few minutes prior to injection of atropine/HI-6 and at approximately 1, 2, 3, 4, 5, 6, 8, 12, 16, 20, 40, 60, 80, 120, 180, 240, 300, and 360 min after injection. Seven-tenths mL of heparinized physiologic saline (30 units/mL) was used as a block in the three-way stopcock and indwelling catheter to prevent clotting of blood during the longer intervals between blood collections. The heparin block was removed by withdrawing a 1-mL volume before drawing the 10-mL blood sample for analysis. Five mL of the 10-mL blood sample drawn using a 10-mL disposable syringe was immediately placed in a prelabeled heparinized glass vacutainer® (Becton Dickinson). The other 5 mL was placed in a prelabeled 13-mL polypropylene tube with cap. This tube was placed on its side and the blood allowed to clot at room temperature for at least one hr. Sheep were removed from slings after the 120-min blood samples were drawn and allowed access to feed and water. Catheters were left in place until after the six-hr blood samples were drawn.

The heparinized blood samples were transferred to labeled polypropylene tubes and centrifuged at approximately 1500 X G for 15 min. Then, the plasma was removed with pipettes, put into labeled polypropylene tubes, and frozen at approximately -70 C until assayed for HI-6. Analyses for HI-6 concentration were conducted at the MREF using a high performance liquid chromatography (HPLC) technique (MREF SOP 89-62). After the blood samples in the non-heparinized tubes had clotted, the blood clots were gently separated from the sides of the tubes with applicator sticks. The tubes were then centrifuged at approximately 800 X G for 15 min and the serum was pipetted into labeled polypropylene tubes and frozen at approximately -70 C. Serum samples were hand-carried to the laboratory of Dr. Larry Miller at Battelle's Columbus site for determination of atropine concentrations, using radioimmunoassay (RIA) techniques (SOP Number: TOX VI-014-00).

The study used a cross-over design balanced for sequence of injection, day of testing effects, and residual effects. Once blood concentrations of atropine and HI-6 were determined, concentrations as a function of time, maximum concentrations, times to maximum concentrations, areas under the blood concentration-time curves from 0 to 6 hr, absorption and elimination rate constants, and apparent volumes of distribution were estimated using the pharmacokinetic model which best represented the data.

Pharmacokinetic parameters for atropine and HI-6 administered by both injection techniques were compared to determine any significant ($P < 0.05$) differences.

Thirty-two separate pharmacokinetic analyses were performed - serum atropine following use of both injection techniques in eight sheep, and plasma HI-6 for both injection techniques in eight sheep. Although blood sampling times were established, it was not always possible to draw samples exactly at desired times, usually because of problems obtaining blood flow in the catheters. Times at which blood samples were actually obtained were recorded for each animal at each sampling time, and pharmacokinetic parameters were estimated using the actual times of blood collection. Atropine concentrations less than 1 ng/mL, the limit of reliable quantification, were considered as zero for the pharmacokinetic analyses. The quantitative limit for HI-6 was 2.5 μg/mL, and values below this concentration were also considered as zero for pharmacokinetic analyses.

The objective of the statistical analysis of the pharmacokinetic data was to determine if any significant differences existed among the HI-6 and atropine pharmacokinetic parameters estimated for the two different injection techniques. Empirical data for C_{max}, t_{max}, and AUC_{0-360}, i.e., the actual highest blood concentration measured, the actual sampling time of this highest concentration, and the area under the measured blood concentrations over time curve to 360 min derived by the trapezoidal method, and the same pharmacokinetic parameters predicted by models were statistically evaluated. The correlations between empirical and model estimates were determined to assess the "goodness of fit" of the models.

Pharmacokinetic parameters were analyzed to determine if there were any effects due to injection technique or week of testing, and to assess the variability in the pharmacokinetic parameters among the animals. Experiments in which the same animal is tested on multiple occasions using different treatment regimens on different testing days are called cross-over designs. By using a cross-over design, comparisons between the pharmacokinetic parameters of different injection techniques can be made on an individual animal basis. Controlling for the animal-to-animal variability by using each animal as its own control provides more precise comparisons between injection

techniques. Special considerations may arise because the effects of a treatment administered one test period may carry over to the next test period (residual effect). Therefore, an animal's blood levels may be affected directly by the most recent treatment and also by a residual effect from the previous treatment. A relatively long washout and recovery period between dosing was used to prevent residual effects.

An analysis of variance appropriate for cross-over designs was conducted for each empirical and model-based estimated pharmacokinetic parameter to assess the statistical significance of the effects of interest. The effects included in the analysis of variance are given in the following equation for a generic pharmacokinetic parameter Y:

$$Y = \mu + \beta + \gamma + \tau + \epsilon$$

where μ = average value of the pharmacokinetic parameter,

β = effect of animal,

γ = effect of week of testing,

τ = direct effect of the autoinjector used that week, and

ϵ = uncontrolled variation within an animal.

Sheep were observed at 20 min after injection of the atropine/HI-6 for signs of atropinization, including pupillary size changes and response to light and changes from preinjection heart rate. Muscle coordination in ambulation was evaluated at 120 min after injection of atropine/HI-6 when sheep were removed from slings.

2.2.5 GD Aging Studies

This portion of the task was designed to determine the rate of aging [i.e., the rate of dealkylation of acetylcholinesterase (AChE)-bound GD and formation of irreversible GD-AChE binding] of GD bound to AChE from sheep red

blood cells (sRBC) using a method developed in Task 38-36 of a previous contract. The assay is based upon the percent of AChE dephosphonylated (reactivated) by HI-6 added at different times of GD-AChE incubation, with aging rate defined as the slope of the log percent reactivation versus time plot. This method is based upon the work of Harris, et al.[11] and automated using a Cobas FARA (Roche Diagnostic Systems, Nutley, NJ) centrifugal autoanalyzer.

Blood samples from ten different sheep were collected and the aging rates of GD-inhibited AChE were determined to measure animal-to-animal variability. Approximately 10 mL of blood was collected from each sheep in a vacutainer containing heparin as an anticoagulant. The AChE present on the membranes of the sRBC was made soluble with Triton X-100 in accordance with MREF SOP 38-46 (Appendix B). The AChE activity present in the different samples was adjusted to approximately the same baseline level, and the inhibition of AChE activity for various concentrations of GD was determined. From the inhibition data, a GD concentration was selected for use in aging studies. The HI-6 concentration used was fixed at 800 μM, as this concentration is known to be effective in reactivating fetal bovine serum AChE.[12]

Procedures were performed in three steps:

1. AChE Preparation: Blood samples from sheep were collected in heparinized vacutainers and then centrifuged at 8,800 X G for ten min. The sRBC were osmotically lysed by resuspending the red cell pellet in distilled deionized water to form membrane ghosts which were isolated by centrifugation on the day of blood collection (MREF SOP-88-46). Samples of AChE were prepared by dissolving the sRBC membrane ghosts in a 1 percent Triton X-100 in physiologic saline solution. The AChE samples were then aliquoted into 1.7-mL polypropylene tubes and stored at -10 C until used. The use of AChE from membrane ghosts eliminates the high background absorbance readings found if heme is present, allows concentration of sample AChE activity, and increases the sensitivity of the measurements.

2. GD-Induced Inhibition of AChE Activity: An aliquot of AChE from RBCs of each sheep was thawed, assayed for AChE activity, and the AChE activity diluted to approximately 20 mUnits per mL with the 1 percent Triton X-100 in physiologic saline solution. GD, at various concentrations, was incubated with the AChE samples for five minutes prior to determining AChE activity using the centrifugal analyzer. Incubation and AChE activity determinations were performed at 23 C. Slope and y-intercept values were calculated, using regression analyses of log GD concentration (x-axis) versus AChE inhibition values between 15 percent and 85 percent. The GD concentration required to produce a given amount of AChE inhibition was estimated from the regression line equation and individual slope and y-intercept values for the experimental conditions used. The IC_{50} value, that GD concentration required to produce 50 percent inhibition of AChE under the experimental conditions, was calculated for each animal.
3. Aging of GD-Inhibited AChE: GD was incubated with AChE samples for various lengths of time to allow different degrees of aging. After incubation, HI-6 was added to a final concentration of 800 μM, and the amount of HI-6-induced AChE reactivation was determined. The aging rate of GD-inhibited AChE is described as a first-order process and graphical presentation of log percent reactivation versus length of incubation yields a linear plot.[12] Aging rate, as defined by the slope of the semi-logarithmic plot, was determined at 23 C and at 37 C.

3.0 RESULTS

3.1 Chemistry

USAMRICD reported that wet/dry autoinjectors (ASTRA) delivered 1.86 mg atropine and 460 mg HI-6 in a total volume of 3.15 mL. The MKI system is designed to deliver 2 mg atropine sulfate equivalents using the Atropen

(Lot No. RU7213, Manuf. date 5/85) and 600 mg 2 PAM by the 2-PAM autoinjector (Lot No. RU8243, Manuf. date 9/85).

Chemical analyses of GD dosing solutions (MREF SOP 88-31) from LD_{50} and efficacy studies confirmed concentrations within ± 6 percent of 1.7 mg/mL on all days except two - one analysis at 88 percent of target and one analysis at 82 percent. The stability of GD solutions is dependent upon temperature and handling, and the solution demonstrating only 82 percent of target concentration was not analyzed until 6 days after dosing.

Although some incorrect assumptions were made, as discussed in 3.3 Treatment Efficacy Evaluation, chemical analyses of daily dosing solutions demonstrated an atropine dose within ± 8 percent of the target dose (MREF SOP 89-55) and an HI-6 dose within ± 5 percent of the target dose (MREF SOP 89-64). For pharmacokinetic studies, chemical analyses of dosing solutions confirmed HI-6 doses within 5 percent of target and atropine doses within 7 percent of target, except for the first day of pharmacokinetic studies when the atropine concentration was analyzed at 84 percent of target.

3.2 GD LD_{50} Estimation

A modified up-down type experiment, challenging one or two sheep per day, was conducted to estimate the 48-hr GD LD_{50} in the present population of untreated sheep. Based on historic information on the slope of the GD dose-lethal response curve and analyses of data as they were obtained, the best doses for challenging succeeding animals were selected to estimate the 48-hr GD LD_{50} most efficiently. A total of five sheep were injected with GD, on three different dosing days, and given no therapy. The doses delivered and results are presented in Table 1. Dose-response models were fitted to the data from the five sheep of Task 89-06 and the 80 animals used in Task 88-38, and 48-hr GD LD_{50}s were estimated for each group of untreated sheep. The two LD_{50}s were not statistically different (at the 0.05 significance level) and the data from the two groups of untreated sheep were pooled to form one group. The 48-hr GD LD_{50} in this pool of untreated sheep was calculated to be 6.6 µg/kg.

TABLE 1. RESULTS OF TASK 89-06 GD LD_{50} STUDY IN SHEEP

Animal Number	Weight (kg)	GD Dose (μg/kg)	Time of Death
28	69.3	5.6	-
43	73.0	5.7	-
51	74.1	6.3	11 min
6	60.9	7.3	72 min
40	64.5	7.7	75 min

3.3 Treatment Efficacy Evaluation

On the first day of treatment efficacy testing, an atropine stock solution was used as diluent in preparing an atropine/HI-6 admixed solution. HI-6 was weighed and dissolved in the atropine stock solution in a manner to deliver 1.86 mg atropine free base and 460 mg HI-6 in 3.15 mL of solution. Seventeen sheep were given various GD doses, selected by the study statistician in collaboration with the study director. At one min after GD injection, five of these sheep were treated with three MKI autoinjectors each, seven were treated with three wet/dry autoinjectors each, and each of the remaining five sheep was treated with three 3.15-mL injections via syringe of the atropine/HI-6 solution prepared that morning. It was assumed that each MKI Atropen injected 2 mg of atropine sulfate equivalents, and each wet/dry autoinjector and each prepared atropine/HI-6 solution injection gave a dose of 2.2 mg of atropine sulfate equivalents. Based on this assumption, additional atropine sulfate in a 15 mg/mL commercial solution was injected separately to provide a total atropine sulfate treatment dose of 0.5 mg/kg.

Following the first day of efficacy testing, a report on the operational evaluation of the wet/dry autoinjector was received from USAMRICD.[13] From this report it was determined that the average volume delivered with the wet/dry autoinjector was 3.05 mL and the average atropine dose of 1.86 mg was in atropine sulfate equivalents rather than atropine free base as had been assumed. Therefore a new formulation of the atropine stock solution had to be prepared and the amount of HI-6 to be added changed to provide dosing day solutions containing 1.86 mg atropine sulfate equivalents and 460 mg of HI-6 in a 3.05 mL volume. This did not change the total dose of HI-6 delivered, although it did decrease the total volume delivered by 0.3 mL, and did increase slightly the supplemental dose of atropine sulfate needed. The increase in the supplemental dose of atropine was calculated, using the corrected values for atropine content, for the seven sheep dosed with the wet/dry autoinjector on the first day of efficacy testing to be 1 mg or 0.07 mL of the 15 mg/mL supplemental atropine solution. The body weight range for these seven sheep was 66.8-77.7 kg, and the supplemental dose of atropine was designed to deliver a total atropine sulfate dose of 0.5 mg/kg. Thus, 1 mg

error in the dose injected in the lightest sheep decreased the total dose of atropine sulfate delivered by 3 percent, and results of this error are probably inconsequential.

Procedures similar to those used on the first day of efficacy testing were performed on the second day with only the atropine/HI-6 via syringe dose formulation being changed. After two days of efficacy testing, a total of 14 sheep had been treated with wet/dry autoinjectors, ten with MKI autoinjectors, and ten with atropine/HI-6 via syringe. Although the estimated LD_{50}s of treated sheep were approximately twice the LD_{50} of untreated sheep, there were no statistical differences ($P < 0.05$) between the 48-hr GD LD_{50}s estimated for the treatment groups. As stated in the protocol, because there was not a significant difference in LD_{50} values, the USAMRICD Task Area Manager and the Contracting Officer's Representative were contacted and informed of these findings. As a result, it was decided to use an additional treatment group: atropine administered via three MKI Atropens plus HI-6 (a total of 1,380 mg delivered via three syringes at a volume of 3.05 mL per syringe) followed by the supplemental dose of atropine. On the third day of efficacy testing, five sheep were challenged with GD and treated with atropine/2-PAM from three MKI autoinjectors, five were challenged and treated with the HI-6/Atropen combination, and five sheep were challenged and treated with three syringes, each containing the atropine/HI-6 admixture. All sheep were given the supplemental dose of atropine. The same treatment regimens used on the third day of testing were also used on the fourth day of efficacy testing.

After four days of testing, because the 48-hr GD LD_{50}s estimated for the HI-6 therapies were not statistically significantly greater than that calculated for the atropine/2-PAM MKI therapy, an additional treatment group was added to test the treatment efficacy of atropine alone (three injections with 1.86 mg atropine sulfate equivalents per injection plus supplemental atropine). On the fifth day of efficacy testing, of the ten sheep challenged, five were treated with atropine alone and five were treated with the MKI autoinjectors. All sheep were given supplemental atropine. On the sixth and final day of efficacy testing, the treatment efficacies of atropine/HI-6 given by three wet/dry autoinjectors (4 sheep), equivalent doses of atropine/HI-6

given by syringe (4 sheep), atropine/2-PAM given by three MKI autoinjectors (4 sheep), and atropine alone given by syringe (5 sheep) were compared. As on previous days, all treatments were given with sufficient quantities of supplemental atropine to deliver 0.5 mg/kg body weight.

The treatment groups studied in this task, their abbreviated group names, and number of animals tested in each treatment group are displayed in Table 2. The results of treatment efficacy testing are presented in Table 3.

3.3.1 Statistical Analyses

The statistical analyses of results of this study rely on a number of assumptions. It was assumed that the probit model, with no background lethality, provided a reasonable approximation of the GD dose-lethality response relationship, at least between the 10th and 90th percentiles. It was also assumed that the slopes of the dose-response relationships for each group of sheep studied in this task (Untreated, Atr/Syr, 2-PAM/MKI, HI-6/Syr, HI-6/Wet-Dry, and HI-6/Atropen) could be approximated by a single slope. It was further assumed that the sheep were drawn from a single population, with no drift in response over time. The absence of a time drift in response permits the use of a stagewise dose allocation strategy, as opposed to testing all animals simultaneously.

3.3.1.1 Analysis of 48-Hr Lethality Data

Data from sheep given virtually no therapy in Task 88-38 and data from Task 89-06 were used to estimate the 48-hr GD LD_{50}s for untreated sheep and for treatments of atropine alone, atropine/2-PAM, and atropine/HI-6 with different delivery techniques. A probit dose-response model in log GD dose was fitted to the 48-hr lethality results for the six groups of sheep. This model assumed that the dose-response relations for the six groups of sheep had a common slope but different intercepts. The historic data from Task 88-38 was included to estimate the 48 hr-GD LD_{50} for untreated sheep, and to increase the precision of the slope estimate, thereby increasing the precision of the 48-hr GD LD_{50} estimates for the other treatment groups. The parameter

TABLE 2. TREATMENT GROUPS USED IN TASK 89-06

Abbreviated Treatment Name	Description of Treatment[a]	Number of Sheep
Atr/Syr	5.6 mg atropine delivered by 3 syringes	10
2-PAM/MKI	6.0 mg atropine plus 1,800 mg 2-PAM Cl given with 3 MKI autoinjectors	29
HI-6/Syr	5.6 mg atropine plus 1,380 mg HI-6 given admixed in 3 syringes	24
HI-6/Wet-Dry	5.6 mg atropine plus 1,380 mg HI-6 given with 3 wet/dry autoinjectors	18
HI-6/Atropen	6.0 mg atropine given with 3 MKI Atropens plus 1,380 mg HI-6 given with 3 syringes	10

[a] Treated sheep received an injection of atropine sulfate to supplement that given in autoinjectors or syringes to give each animal a total dose of 0.5 mg/kg.

TABLE 3. TEN-HR AND 48-HR LETHALITY RESULTS, AND TIMES TO DEATH FOR SIX GROUPS OF SHEEP: UNTREATED, ATR/SYR, 2-PAM/MKI, HI-6/SYR, HI-6/WET-DRY, AND HI-6/ATROPEN

Task	Treatment	Animal I.D.	Body Weight (kg)	GD Dose (μg/kg)	48 Hr Lethality	10 Hr Lethality	Time to Death[a] (min)
88-38	Untreated	32	60.0	4.2	Lived	Lived	-
		20	62.2	4.4	Lived	Lived	-
		36	60.0	4.5	Lived	Lived	-
		3	60.0	4.5	Lived	Lived	-
		66	58.2	5.2	Lived	Lived	-
		22	64.8	5.3	Lived	Lived	-
		18	66.4	5.4	Lived	Lived	-
		28	61.8	5.5	Lived	Lived	-
		14	63.4	5.5	Lived	Lived	-
		38	63.0	5.6	Died	Lived	791
		60	63.2	5.6	Died	Lived	776
		74	66.4	5.7	Lived	Lived	-
		5	58.2	5.8	Lived	Lived	-
		30	60.9	5.8	Lived	Lived	-
		31	62.7	5.8	Lived	Lived	-
		43	57.7	5.9	Lived	Lived	-
		50	59.1	5.9	Lived	Lived	-
		44	60.0	6.0	Lived	Lived	-
		24	59.1	6.0	Lived	Lived	-
		35	61.8	6.1	Lived	Lived	-
		45	65.0	6.1	Died	Died	60
		6	60.9	6.1	Lived	Lived	-
		78	60.2	6.1	Lived	Lived	-
		42	64.1	6.2	Lived	Lived	-
		64	60.5	6.2	Lived	Lived	-
		53	62.7	6.2	Died	Died	83
		57	69.1	6.3	Lived	Lived	-

TABLE 3.
(Continued)

Task	Treatment	Animal I.D.	Body Weight (kg)	GD Dose (μg/kg)	48 Hr Lethality	10 Hr Lethality	Time to Death (min)
88-38	Untreated	73	62.3	6.3	Lived	Lived	-
		72	64.1	6.4	Died	Died	60
		56	66.8	6.4	Lived	Lived	-
		62	65.9	6.5	Lived	Lived	-
		65	63.6	6.5	Died	Died	55
		81	58.0	6.5	Lived	Lived	-
		63	60.9	6.6	Lived	Lived	-
		76	58.0	6.6	Died	Died	72
		47	64.1	6.6	Died	Died	54
		16	70.0	6.6	Died	Died	94
		79	63.6	6.7	Died	Died	41
		25	62.3	6.7	Lived	Lived	-
		17	72.7	6.8	Died	Died	46
		41	63.6	7.0	Died	Died	13
		59	63.6	7.0	Died	Died	163
		80	66.4	7.0	Died	Lived	755
		19	57.7	7.0	Died	Died	77
		71	66.8	7.0	Died	Died	57
		58	65.0	7.0	Died	Died	78
		10	60.0	7.2	Lived	Lived	-
		55	63.0	7.2	Died	Died	112
		54	61.4	7.2	Died	Died	70
		8	71.1	7.2	Died	Died	51
		7	70.9	7.3	Died	Died	90
		11	62.3	7.3	Lived	Lived	-
		61	60.5	7.4	Died	Died	15
		29	70.2	7.5	Died	Died	23
		70	60.0	7.5	Died	Died	41

TABLE 3.
(Continued)

Task	Treatment	Animal I.D.	Body Weight (kg)	GD Dose (µg/kg)	48 Hr Lethality	10 Hr Lethality	Time to Death (min)
88-38	Untreated	4	72.0	7.5	Died	Lived	734
		82	63.6	7.5	Died	Died	75
		46	62.3	7.6	Lived	Lived	-
		77	60.0	7.6	Lived	Lived	-
		37	61.4	7.6	Lived	Lived	-
		27	68.4	8.0	Died	Died	14
		75	63.6	8.0	Died	Died	17
		68	63.6	8.0	Lived	Lived	-
		51	66.8	8.0	Died	Died	65
		69	63.6	8.1	Died	Died	54
		52	61.4	8.1	Died	Died	31
		49	62.3	8.2	Died	Died	34
		33	60.0	8.3	Died	Died	84
		48	60.7	8.3	Died	Died	62
		15	69.3	8.4	Died	Died	51
		23	60.7	8.7	Lived	Lived	-
		40	67.5	8.7	Died	Died	[illegible]
		21	59.5	11.0	Died	Died	2[illegible]
		26	60.0	11.2	Died	Died	36
		9	60.9	13.3	Died	Died	74
		1	62.7	13.6	Died	Died	31
		12	60.5	16.7	Died	Died	75
		39	63.0	16.8	Died	Died	39
		2	62.0	24.9	Died	Died	58
		34	62.0	25.0	Died	Died	30
89-06	Untreated	28	69.3	5.6	Lived	Lived	-
		43	73.0	5.7	Lived	Lived	-
		51	74.1	6.3	Died	Died	11

TABLE 3.
(Continued)

Task	Treatment	Animal I.D.	Body Weight (kg)	GD Dose (μg/kg)	48 Hr Lethality	10 Hr Lethality	Time to Death (min)
89-06	Untreated	6	60.9	7.3	Died	Died	72
		40	64.5	7.7	Died	Died	75
89-06	Atr/Syr	102	73.4	7.0	Died	Lived	1,140
		98	66.8	8.1	Died	Lived	1,470
		111	78.0	9.1	Died	Died	154
		130	80.2	9.5	Died	Lived	1,170
		135	69.1	10.1	Lived	Lived	-
		10	98.2	11.4	Died	Died	174
		141	65.9	11.9	Died	Died	238
		31	81.8	12.6	Died	Lived	[b]600
		26	100.0	13.5	Died	Died	12
		136	72.3	16.5	Died	Died	69
89-06	2-PAM/MKI	121	76.4	9.0	Lived	Lived	-
		122	72.5	9.3	Lived	Lived	-
		110	82.3	10.1	Died	Lived	1,170
		83	81.8	10.4	Lived	Lived	-
		99	64.3	10.7	Died	Died	204
		105	66.8	11.2	Lived	Lived	-
		57	72.7	11.5	Lived	Lived	-
		95	65.9	11.9	Lived	Lived	-
		146	77.7	12.0	Died	Died	109
		100	71.6	12.3	Lived	Lived	-
		108	67.5	12.7	Died	Died	77
		38	78.6	12.8	Lived	Lived	-
		106	72.3	12.8	Died	Lived	1,080
		55	90.9	13.5	Died	Died	169
		90	81.8	13.6	Died	Died	7

TABLE 3.
(Continued)

Task	Treatment	Animal I.D.	Body Weight (kg)	GD Dose (μg/kg)	48 Hr Lethality	10 Hr Lethality	Time to Death (min)
89-06	2-PAM/MKI	137	75.5	14.1	Died	Died	201
		54	71.6	14.2	Lived	Lived	-
		143	61.4	14.2	Lived	Lived	-
		48	79.3	14.5	Died	Died	204
		88	70.5	15.1	Died	Lived	1,050
		33	71.8	15.6	Lived	Lived	-
		9	72.7	15.9	Died	Died	44
		125	68.6	15.9	Died	Lived	1,110
		32	63.6	17.2	Died	Died	10
		11	79.5	17.4	Died	Died	52
		21	67.3	17.7	Lived	Lived	-
		14	79.5	18.8	Died	Died	111
		17	71.1	19.5	Died	Lived	1,020
		52	70.5	22.8	Died	Died	254
89-06	HI-6/Syr	29	85.5	10.8	Lived	Lived	-
		124	76.4	11.1	Lived	Lived	-
		41	84.1	11.3	Lived	Lived	-
		115	65.0	11.7	Lived	Lived	-
		94	70.5	12.0	Lived	Lived	-
		50	73.9	12.8	Died	Died	204
		133	80.0	12.8	Lived	Lived	-
		145	87.5	13.5	Died	Lived	660
		56	78.0	13.9	Died	Died	101
		107	75.5	14.0	Died	Died	66
		3	67.0	14.1	Died	Lived	1,020
		103	67.3	14.5	Died	Died	64
		49	67.3	14.8	Died	Died	48
		139	64.5	15.1	Lived	Lived	-

TABLE 3.
(Continued)

Task	Treatment	Animal I.D.	Body Weight (kg)	GD Dose (μg/kg)	48 Hr Lethality	10 Hr Lethality	Time to Death (min)
89-06	HI-6/Syr	5	80.5	15.7	Died	Lived	990
		16	73.9	16.0	Lived	Lived	-
		45	75.0	16.1	Died	Died	46
		113	80.2	16.5	Died	Died	180
		128	69.3	17.0	Lived	Lived	-
		47	78.9	17.2	Died	Died	62
		131	71.8	18.8	Lived	Lived	-
		2	68.2	19.6	Died	Lived	1,020
		144	78.2	21.1	Died	Died	54
		42	69.5	25.9	Died	Died	159
89-06	HI-6/Wet-Dry	20	69.1	11.0	Lived	Lived	-
		1	71.6	11.2	Lived	Lived	-
		91	77.3	12.5	Lived	Lived	-
		22	77.3	12.9	Lived	Lived	-
		97	78.2	14.1	Lived	Lived	-
		37	65.0	14.5	Died	Died	50
		114	86.4	15.1	Died	Died	45
		19	69.5	15.6	Lived	Lived	-
		4	78.2	15.9	Died	Died	201
		8	77.7	17.1	Died	Lived	1,410
		12	74.8	17.4	Lived	Lived	-
		118	75.5	18.3	Died	Died	240
		34	79.5	19.0	Died	Lived	990
		35	75.9	19.5	Died	Lived	960
		7	72.7	19.6	Died	Lived	1,020
		46	86.4	22.7	Died	Died	38
		53	66.8	25.3	Died	Died	83
		18	67.0	25.9	Died	Died	40

TABLE 3.
(Continued)

Task	Treatment	Animal I.D.	Body Weight (kg)	GD Dose (μg/kg)	48 Hr Lethality	10 Hr Lethality	Time to Death (min)
89-06	HI-6/Atropen	120	68.9	11.5	Lived	Lived	-
		134	74.1	15.0	Lived	Lived	-
		96	68.2	15.9	Died	Died	188
		126	64.3	17.1	Died	Died	198
		138	65.4	17.8	Lived	Lived	-
		132	65.9	19.0	Died	Died	222
		101	62.3	19.0	Died	Lived	1,050
		36	89.5	20.1	Lived	Lived	-
		119	62.3	22.8	Died	Died	307
		109	71.1	24.1	Died	Died	90

(a) For data from Task 89-06, since sheep were not observed continuously, times to death beyond 10 hr were rounded to the nearest half hr preceding the time recorded on clinical observations sheets.

(b) Although the time to death for sheep number 31 is entered as 500 min, the actual time of death is not known and this animal was not considered as dead in the 10-hr lethality analyses.

estimates and results from fitting the common slope model to the 48-hr lethality data are summarized in Table 4. The estimate of the common slope is 9.61, with a standard deviation of 1.59. Graphs of the probit dose-response models estimated for each group of sheep based on the common slope model are displayed in Figure 1.

In addition to fitting a common slope model to the combined 48 hr lethality data from the six groups of sheep, separate probit models were fitted to each group. A hypothesis test of the adequacy of the common slope assumption was performed by comparing the fits from the common slope and separate slopes models. Based on the results of a log-likelihood ratio test, the null hypothesis of a common slope for the six groups of sheep was not rejected at the 5 percent significance level. While this does not prove that the common slope assumption is correct, it does show that there is no evidence in the data contrary to the common slope assumption.

The estimated parameters from the common slope model were used to calculate the 48-hr GD LD_{50} and protective ratio (PR) for each group of sheep. For each group of treated sheep, the PR was calculated as the ratio of the 48-hr GD LD_{50} estimated for the treated sheep to the 48-hr GD LD_{50} estimated for untreated sheep. The estimated 48-hr GD LD_{50} and PR for each group of sheep are presented in Table 5. While the PRs estimated for 2-PAM/MKI, HI-6/Syr, HI-6/Wet-Dry, and HI-6/Atropen were determined to be statistically significantly greater than 1 (at the 5 percent significance level), the PR estimated for Atr/Syr was determined to not be statistically different from 1. Pairwise comparisons were conducted between the 48-hr GD LD_{50}s estimated for the five groups of treated sheep. The results of the statistical comparisons (at the 5 percent significance level) may be summarized as follows:

- The 48-hr GD LD_{50} estimated for Atr/Syr with no oxime therapy was determined to be significantly less than the 48-hr GD LD_{50}s estimated for 2-PAM/MKI, HI-6/Syr, HI-6/Wet-Dry, and HI-6/Atropen.

- There were no statistically significant differences between the 48-hr GD LD_{50}s estimated for HI-6/Syr, HI-6/Wet-Dry, and HI-6/Atropen.

TABLE 4. PARAMETER ESTIMATES AND MODEL RESULTS FROM FITTING COMMON SLOPE PROBIT DOSE-RESPONSE MODEL TO THE 48-HR LETHALITY RESULTS FROM SIX GROUPS OF SHEEP

Non-Linear Least Squares Summary Statistics — Dependent Variable (48-Hr Lethality)

Source	DF	Weighted SS	Weighted MS
Regression	7	239,632.21	34,233.17
Residual	169	165.50	0.98
Uncorrected Total	176	239,797.72	
(Corrected Total)	175	502.42	
Sum of Loss		176.97	

Parameter[a]	Estimate	Asymptotic Standard Error	Asymptotic 95 Percent Confidence Interval Lower	Upper
B1	9.61	1.59	6.47	12.74
B01	- 2.88	1.32	- 5.48	- 0.27
B02	- 3.17	1.62	- 6.37	- 0.03
B03	- 5.62	1.80	- 9.17	- 2.06
B04	- 6.00	1.86	- 9.67	- 2.33
B05	- 6.28	1.93	-10.09	- 2.47
B06	- 6.75	2.03	-10.77	- 2.73

(a) B1 is the estimate of the common slope.
B01 is the intercept for untreated sheep from Task 88-38 and Task 89-06.
B02 is the intercept for the Atr/Syr treated group.
B03 is the intercept for the 2-PAM/MKI treated group.
B04 is the intercept for the HI-6/Syr treated group.
B05 is the intercept for the HI-6/Wet-Dry treated group.
B06 is the intercept for the HI-6/Atropen treated group.

FIGURE 1. PROBIT DOSE-RESPONSE MODELS FOR 48-HR LETHALITY OF SHEEP CHALLENGED WITH GD AND RECEIVING VARIOUS TREATMENTS

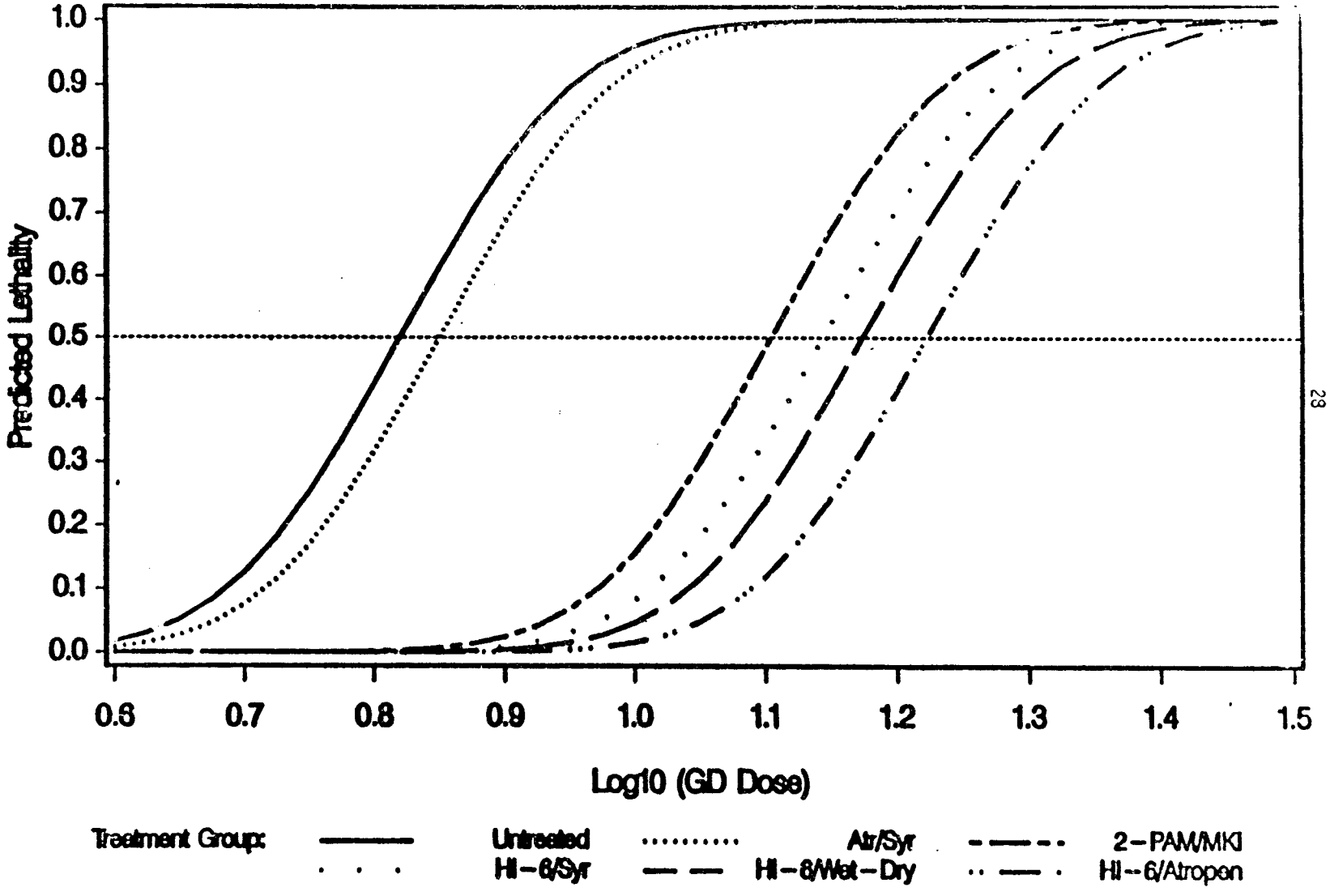


TABLE 5. ESTIMATED 48-HR GD LD_{50}s AND PROTECTIVE RATIOS FOR SIX GROUPS OF SHEEP

Treatment	N	48-Hr GD LD_{50} (µg/kg)		Protective Ratio[a]	
		Estimate	95 Percent C.I.	Estimate	95 Percent C.I.
Untreated	85	6.6	(6.1, 7.1)	1.00	--
Atr/Syr	10	7.1	(5.0, 9.5)	1.07	(0.79, 1.46)
2-PAM/HKI	29	12.7	(11.1, 14.5)	1.93	(1.67, 2.23)
HI-6/Syr	24	14.0	(12.1, 16.0)	2.11	(1.81, 2.46)
HI-6/Wet-Dry	18	15.0	(12.5, 17.7)	2.26	(1.89, 2.71)
HI-6/Atropen	10	16.7	(13.3, 20.7)	2.53	(2.02, 3.16)

[a] 48-hr GD LD_{50} for treated sheep divided by 48-hr GD LD_{50} for untreated sheep.

- While the 48-hr GD LD_{50} estimated for 2-PAM/MKI was determined to not be significantly less than the 48-hr GD LD_{50}s estimated for HI-6/Syr and HI-6/Wet-Dry, it was determined to be statistically significantly less than the 48-hr GD LD_{50} estimated for HI-6/Atropen. The 95 percent confidence interval for the ratio of the 48-hr GD LD_{50} estimated for HI-6/Atropen to the 48-hr GD LD_{50} estimated for 2-PAM/MKI was 1.03 to 1.68. Because of the large number of statistical comparisons conducted at the 5 percent significance level, the statistical significance of this result must be interpreted cautiously.

A second approach to conducting hypothesis tests was utilized to determine if the 48-hr GD LD_{50}s were statistically significantly different for the three Atr/HI-6 treatments. This test was based on the asymptotic chi-square distribution of the log-likelihood ratio test statistic. The resulting value of the test statistic was 1.9. Regarding 1.9 as a value from a chi-square distribution with 2 degrees of freedom, treatments were not statistically different at the 10 percent significance level. Therefore, the data from the three HI-6 regimens were combined into one group of animals, and a probit dose-response model in log dose was fitted to four groups. This model assumed a common slope, but different intercepts for treatment groups: Untreated, Atr/Syr, 2-PAM/MKI, and HI-6. The estimate of the 48-hr GD LD_{50} for the combined HI-6 therapies was 14.7 µg/kg, with a 95 percent confidence interval of 13.2 to 16.3 µg/kg. The estimated PR for the combined HI-6 therapies was 2.23 with a 95 percent confidence interval of 1.97 to 2.53. The ratio of the 48-hr GD LD_{50} estimated for the combined HI-6 therapies to the 48-hr GD LD_{50} estimated for 2-PAM/MKI was 1.16, with a 95 percent confidence interval of 0.98 to 1.37.

3.3.1.2 Analysis of 90-Min, 4-Hr, and 10-Hr Lethality Data

While most of the lethalities observed in untreated sheep in Tasks 88-38 and 89-06 occurred shortly after GD exposure, less than half (22/57) of the lethalities observed in treated animals occurred within 2 hr after GD

injection. Consequently, 48-hr lethality may not be the endpoint best suited for discriminating between the efficacies of HI-6 and 2-PAM, and, therefore, lethality at other timepoints was also investigated.

Probit dose-response models in log GD dose were fitted to the data from the six groups of sheep using both 90-min lethality and 4-hr lethality as the measured response. These models assumed that the dose-response relations for the six groups of sheep had a common slope, but different intercepts. Upon analysis, however, there were strong indications of a lack of fit of the common slope model for both 90-min lethality data and 4-hr lethality data, i.e., the hypothesis of a common slope for the GD dose-lethal response relationships of the six groups of animals at these time points was rejected at the 5 percent significance level. Further analysis indicated that the lack of fit for both the 90-min and the 4-hr lethality data resulted from a shallower slope of the GD dose-lethal response relationship estimated for the animals treated with HI-6 or 2-PAM than for the Atr/Syr or untreated sheep.

Time to death data were further examined. Figure 2 displays a plot of the times to death for nonsurvivors in each group of sheep. Results displayed in Figure 2 suggest that a lethality endpoint between 6 and 10 hr might be better suited for discriminating between the efficacies of HI-6 and 2-PAM. Furthermore, in Task 89-12, lethality at 10 hr had been found to be a suitable endpoint for discriminating between the efficacies of 2-PAM and HI-6 in the treatment of GD intoxication in monkeys. Therefore, a probit dose-response model in log GD dose was fitted to the 10-hr lethality data from the six groups of sheep. Again, this model assumed that the dose-response relations for the six groups of sheep had a common slope but different intercepts. Ten-hr lethality data are displayed in the seventh column of Table 3, and parameter estimates and results from fitting the common slope model to this data are summarized in Table 6. The estimate of the common slope is 8.64, with a standard deviation of 1.45. Graphs of the probit dose-response models estimated for each group of sheep based on the common slope model fitted to the 10-hr lethality data are displayed in Figure 3.

FIGURE 2. TIMES TO DEATH OF SHEEP CHALLENGED WITH GD AND RECEIVING VARIOUS TREATMENTS

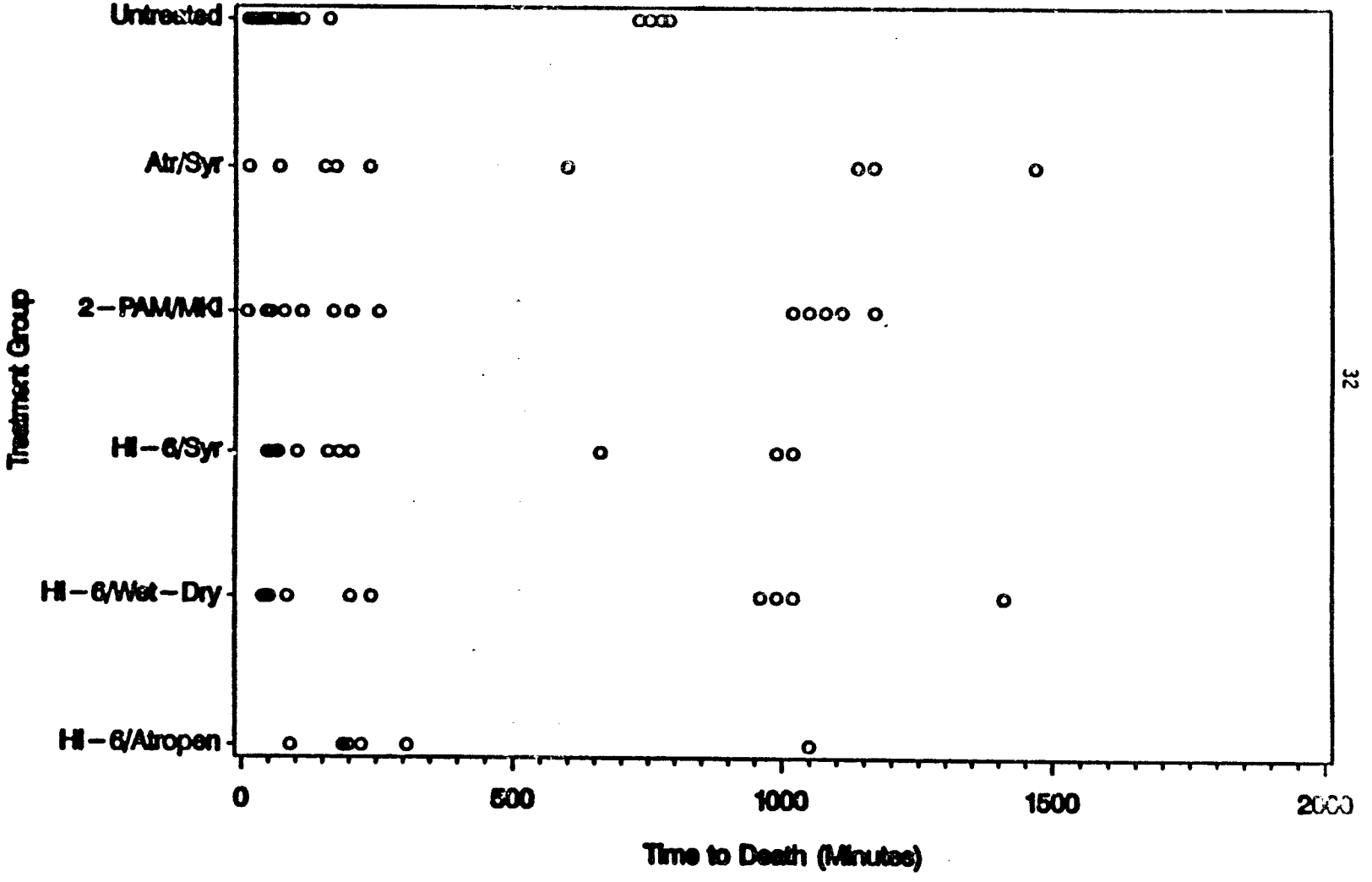


TABLE 6. PARAMETER ESTIMATES AND MODEL RESULTS FROM FITTING COMMON SLOPE PROBIT DOSE-RESPONSE MODEL TO THE 10-HR LETHALITY RESULTS FROM SIX GROUPS OF SHEEP

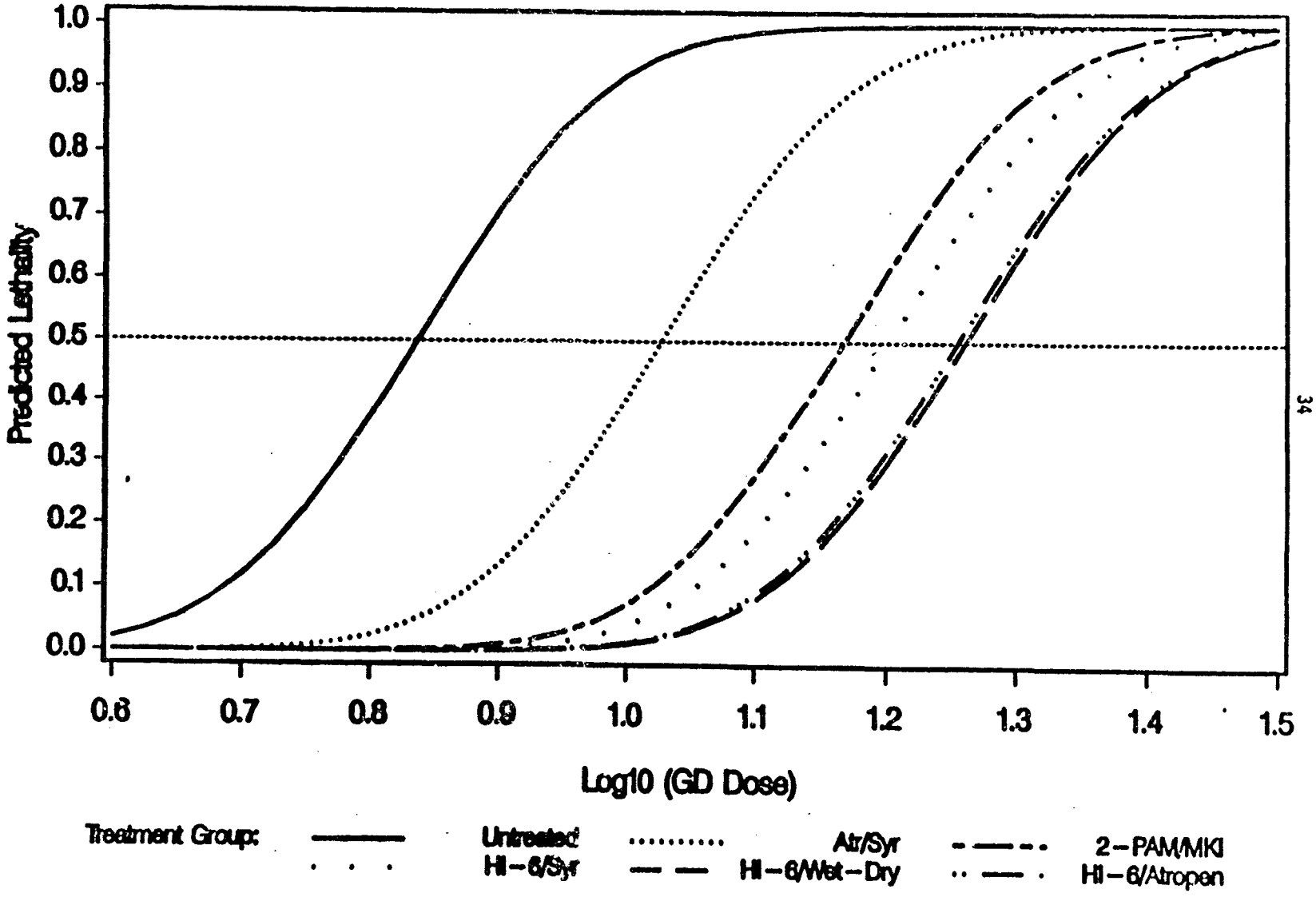
Non-Linear Least Squares Summary Statistics — Dependent Variable (10-Hr Lethality)

Source	DF	Weighted SS	Weighted MS
Regression	7	192,903.85	27,557.69
Residual	169	159.46	0.94
Uncorrected Total	176	193,063.31	
(Corrected Total)	175	735.57	
Sum of Loss		189.41	

Parameter[a]	Estimate	Asymptotic Standard Error	Asymptotic 95 Percent Confidence Interval Lower	Upper
B1	8.64	1.45	5.78	11.49
B01	- 2.23	1.21	-4.62	0.16
B02	- 3.88	1.55	-6.95	- 0.81
B03	- 5.09	1.68	-8.41	- 1.78
B04	- 5.38	1.73	-8.79	- 1.98
B05	- 5.90	1.82	-9.49	- 2.30
B06	- 5.84	1.87	-9.54	- 2.14

[a] B1 is the estimate of the common slope.
B01 is the intercept for untreated sheep from Task 88-38 and Task 89-06.
B02 is the intercept for the Atr/Syr treated group.
B03 is the intercept for the 2-PAM/MKI treated group.
B04 is the intercept for the HI-6/Syr treated group.
B05 is the intercept for the HI-6/Wet-Dry treated group.
B06 is the intercept for the HI-6/Atropen treated group.

FIGURE 3. PROBIT DOSE-RESPONSE MODELS FOR 10-HR LETHALITY OF SHEEP CHALLENGED WITH GD AND RECEIVING VARIOUS TREATMENTS



In addition to fitting a common slope model to the combined 10-hr lethality data from the six groups of sheep, separate probit models were fitted to each group. A hypothesis test of the adequacy of the common slope assumption was performed by comparing the fits from the common slope and separate slopes models. Based on the results of a log-likelihood ratio test, the null hypothesis of a common slope for the six groups of sheep was not rejected at the 5 percent significance level. While this does not prove that the common slope assumption is correct, it does show that there is no evidence in the data contrary to the common slope assumption.

The estimated parameters from the common slope model were used to calculate the 10-hr GD LD_{50} and PR for each group of sheep. For each group of treated sheep, the PR was calculated as the ratio of the 10-hr GD LD_{50} estimated for the treated sheep to the 10-hr GD LD_{50} estimated for untreated sheep. The estimated 10-hr GD LD_{50} and PR for each group of sheep are displayed in Table 7. Estimated PRs were determined to be statistically significantly greater than 1 (at the 5 percent significance level) for all five groups of treated sheep: Atr/Syr, 2-PAM/MKI, HI-6/Syr, HI-6/Wet-Dry, and HI-6/Atropen. Pairwise comparisons were conducted between the 10-hr GD LD_{50}s estimated for the five groups of treated sheep. The results of the statistical comparisons (at the 5 percent significance level) may be summarized as follows:

- The 10-hr GD LD_{50} estimated for Atr/Syr was determined to be statistically less than the 10-hr GD LD_{50}s estimated for 2-PAM/MKI, HI-6/Syr, HI-6/Wet-Dry, and HI-6/Atropen.

- The 10-hr GD LD_{50} estimated for 2-PAM/MKI was determined to not be statistically different from the 10-hr GD LD_{50}s estimated for HI-6/Syr, HI-6/Wet-Dry, and HI-6/Atropen.

- There were no statistical differences among the 10-hr GD LD_{50}s estimated for HI-6/Syr, HI-6/Wet-Dry, and HI-6/Atropen.

TABLE 7. ESTIMATED 10-HR GD LD_{50}s AND PROTECTIVE RATIOS FOR SIX GROUPS OF SHEEP

Treatment	N	10-Hr GD LD_{50} (µg/kg)		Protective Ratio[a]	
		Estimate	95 Percent C.I.	Estimate	95 Percent C.I.
Untreated	85	6.9	(6.3, 7.5)	1.00	--
Atr/Syr	10	10.7	(8.3, 13.7)	1.55	(1.21, 1.99)
2-PAM/MKI	29	14.7	(12.8, 17.2)	2.14	(1.83, 2.51)
HI-6/Syr	24	15.9	(13.7, 18.8)	2.32	(1.96, 2.74)
HI-6/Wet-Dry	18	18.3	(15.2, 22.3)	2.66	(2.18, 3.23)
HI-6/Atropen	10	18.0	(14.1, 22.8)	2.62	(2.06, 3.33)

[a] 10-hr GD LD_{50} for treated sheep divided by 10-hr GD LD_{50} for untreated sheep.

A second approach to conducting hypothesis tests was utilized to determine if the 10-hr GD LD_{50}s were statistically different for the three Atr/HI-6 treatments. This test was based on the asymptotic chi-square distribution of the log-likelihood ratio test statistic. The resulting value of the test statistic was 1.6. Regarded as a value from a chi-square distribution with 2 degrees of freedom, the three Atr/HI-6 treatments were not statistically different at the 10 percent significance level. Therefore, the data from the three HI-6 regimens were combined into one group of animals and a probit dose-response model in log dose was fitted to four treatment groups. This model assumed a common slope, but different intercepts for the following groups: Untreated, Atr/Syr, 2-PAM/MKI, and HI-6. The estimate of the 10-hr GD LD_{50} for the combined HI-6 therapies was 17.1 μg/kg, with a 95 percent confidence interval of 15.3 to 19.3 μg/kg. The estimated PR for the combined HI-6 therapies was 2.49, with a 95 percent confidence interval of 2.17 to 2.85. The ratio of the 10-hr GD LD_{50} estimated for the combined HI-6 therapies to the 10-hr GD LD_{50} estimated for 2-PAM/MKI was 1.16, with a 95 percent confidence interval of 0.97 to 1.38.

3.3.1.3 Analysis of the Times to Death

Because 48-hr or even 10-hr lethality may not be the best endpoint for discriminating between efficacies of HI-6 and 2-PAM, times to death were statistically analyzed. The objective of the statistical analysis of the times to death was to determine if there existed a statistically significant relationship between time to death and GD dose for the six groups of sheep.

Times to death for the animals studied in this task are displayed in the last column of Table 3. Times to death were treated as right censored at 48 hr for animals that survived the 48-hr observation period. This means that the time to death was not known but would have been greater than or equal to the assigned value had the observation period been longer. The natural logarithms (ln) of the times to death were modeled as a linear function of the ln GD doses for six groups of sheep. This model assumed that the relationships between ln time to death and ln GD dose had a common slope but different intercepts. Parameter estimates and results from fitting the common slope model to this data are summarized in Table 8. The estimated parameters

TABLE 8. PARAMETER ESTIMATES AND MODEL RESULTS FOR REGRESSION MODEL OF TIME TO DEATH VERSUS GD DOSE

Variable	DF	Estimate	Standard Error	ChiSquare	P > Chi	Label/Value
B1	1	-5.39	0.77	49.47	0.0001	Slope Coefficient
B01	1	17.29	1.58	119.07	0.0001	Untreated Intercept
B02	1	18.73	1.98	89.09	0.0001	Atr/Syr Intercept
B03	1	21.21	2.10	101.65	0.0001	2-PAM/MKI Intercept
B04	1	21.68	2.18	98.90	0.0001	HI-6/Syr Intercept
B05	1	22.31	2.30	94.50	0.0001	HI-6/Wet-dry Intercept
B06	1	22.87	2.41	90.18	0.0001	HI-6/Atropen Intercept
σ	1	2.47	0.19			Standard Deviation of Times to Death

Estimated Covariance Matrix

B1	B01	B02	B03	B04	B05	B06	σ
0.588	-1.193	-1.395	-1.567	-1.618	-1.691	-1.728	-0.034
-1.193	2.511	2.831	3.184	3.287	3.435	3.510	0.083
-1.395	2.831	3.936	3.718	3.838	4.012	4.099	0.083
-1.567	3.184	3.718	4.426	4.315	4.510	4.608	0.103
-1.618	3.287	3.838	4.315	4.751	4.655	4.756	0.106
-1.691	3.435	4.012	4.510	4.655	5.268	4.972	0.110
-1.728	3.510	4.099	4.608	4.756	4.972	5.799	0.112
-0.034	0.083	0.083	0.103	0.106	0.110	0.112	0.036

of the ln-ln regression model were used to predict the time to death at 2 times the estimated 48-hr GD LD_{50} of untreated sheep (13.2 μg/kg) for each treatment. The predicted times to death at a GD dose of 13.2 μg/kg are displayed in Table 9.

Statistical hypothesis tests were conducted to determine if the predicted times to death at a GD dose of 13.2 μg/kg were significantly different for the six groups of sheep. Based on pairwise comparisons of the natural logarithms of the predicted times to death, the predicted times to death at 13.2 μg/kg GD for Atr/Syr-treated sheep and untreated sheep were determined to be statistically less (at the 5 percent significance level) than the times to death at the same GD dose predicted for 2-PAM/MKI, HI-6/Syr, HI-6/Wet-Dry, and HI-6/Atropen treated sheep. While the predicted time to death at 13.2 μg/kg GD estimated for 2-PAM/MKI treated sheep was not statistically different than the values estimated for each of the HI-6 delivery systems, it was less than the predicted time to death for each HI-6 delivery system.

3.3.1.4 Analyses of Clinical Signs Data

The clinical signs of GD intoxication in the six groups of sheep were also statistically analyzed. Because the current procedures used for monitoring the presence of clinical signs have changed slightly from those used in Task 88-38, only the 96 sheep tested in Task 89-06 were included in the analysis of clinical signs. The objective of the analyses of clinical signs was to determine if there existed statistically significant differences between the onset and duration times of the sublethal responses for the different treatments and delivery systems. Each animal was continuously monitored for clinical signs of GD intoxication during the first 120 min following GD injection, and at decreasing frequencies thereafter. Clinical signs compiled for statistical analyses were onset and cessation times of tremors, convulsions, sternal recumbency, and prostration. Annotated clock times from the clinical signs observation sheets, and letter codes designating the observation interval in which events were observed are given in Table 10 for time to death, and onset and cessation of tremors, convulsions, sternal recumbency, and prostration.

TABLE 9. PREDICTED TIMES TO DEATH AT TWO TIMES THE UNTREATED 48-HR GD LD_{50} FOR SIX GROUPS OF SHEEP

Treatment	N	Predicted Time to Death at 13.2[a] µg/kg GD		
		Estimate (min)	95 Percent C.I. (min)	
Untreated	85	29	10	80
Atr/Syr	10	122	25	592
2-PAM/MKI	29	1,464	544	3,940
MI-6/Syr	24	2,332	770	7,063
MI-6/Wet-Dry	18	4,402	1,169	16,581
MI-6/Atropen	10	7,572	1,331	44,235

[a] 13.2 µg/kg GD is two times the estimated 48-hr GD LD_{50} of untreated sheep.

TABLE 18. CLINICAL OBSERVATIONS FOR ONSET AND CESSATION OF TREMORS, CONVULSIONS, PROSTRATION, AND STERNAL RECUMBENCY

LETTER CODE DESIGNATION (event occurred within this time period after dosing)

A:	0-15 min	E:	60-75 min	I:	2-8 hr
B:	15-30 min	F:	75-90 min	J:	8-24 hr
C:	30-45 min	G:	90-105 min	K:	24-32 hr
D:	45-60 min	H:	105-120 min	L:	32-48 hr

Z: Animal did not die within 48 hr time period.
.: No data
N/A: Not applicable

AA: Event did not occur within +120 min/48 hr [illegible]
BB: Animal's first convulsion occurred +120 min from dosing. Onset is recorded, duration is not recorded
CC: Animal died within 48 hrs. Onset and duration prostration/recumbency are not recorded.
DD: Animal did not live to +120 min. Onset of tremors and convulsions are recorded, duration is not recorded.

Tremors and convulsions (duration) were analyzed through +120 min. from exposure. Prostration and Recumbency were analyzed through +48 hrs from exposure. These signs may extend beyond these time points but are not recorded as such below.

Sheep I.D. Number	Dosing Date	Animal Weight (kg)	Agent Inj. Time	Actual Dose Delivered (µg/kg)	Type Treatment	Time to Death Block Code	Time to Death Noted Time	Duration of Tremors (+1 min to +120 min) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of Convulsions (+1 min to +120 min) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of Sternal/Ventral Recumbency (up to +48 hr) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of Prostration (up to +48 hr) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time
Group #1																							
28	03/14/90	60.3	10:48	5.8	N/A	Z		A	10:51	N	.	A	10:54	D	11:45	G	.	F	.	AA			
46	03/14/90	64.5	11:11	7.7	N/A	E	12:00	A	11:14	DD		C	11:41	DD		CC				CC			
43	03/19/90	73.0	10:36	5.7	N/A	Z		A	10:40	•	.	AA				B	.	B	.	AA			
8	03/19/90	60.9	11:00	7.8	N/A	E	12:20	A	11:12	DD		C	11:44	DD		CC				CC			
61	03/21/90	74.1	10:09	6.8	N/A	A	10:20	A	10:11	DD		A	10:17	DD		CC				CC			

•Tremors continued through +120 min.

Sheep I.D. Number	Dosing Date	Animal Weight (kg)	Agent Inj. Time	Actual Dose Delivered (µg/kg)	Type Treatment	Time to Death Block Code	Time to Death Noted Time	Duration of Tremors (+1 min to +120 min) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of Convulsions (+1 min to +120 min) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of Sternal/Ventral Recumbency (up to +48 hr) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of Prostration (up to +48 hr) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time
Group #1																							
1	04/03/90	71.6	10:57	11.2	Wet/Dry	Z		A	11:02	C	.	AA				AA				AA			
41	04/03/90	64.1	11:02	11.8	Syringe	Z		AA				AA				AA				AA			
57	04/03/90	72.7	11:10	11.5	MKI	Z		A	11:14	B	.	AA				AA				AA			
8	04/03/90	77.7	11:17	17.1	Wet/Dry	J	11:04	A	11:20	B	.	AA				CC				CC			
47	04/03/90	78.9	11:24	17.2	Syringe	E	12:20	A	11:27	DD		A	11:30	D	12:10	CC				CC			
32	04/03/90	63.6	11:30	17.2	MKI	A	11:40	A	11:32	DD		A	11:32	DD		CC				CC			
10	04/03/90	67.0	12:20	25.0	Wet/Dry	C	13:00	A	12:31	DD		A	12:39	DD		CC				CC			
42	04/03/90	69.5	12:30	25.0	Syringe	I	15:15	A	12:30	N	.	A	12:40	D	13:20	CC				CC			
30	04/03/90	70.0	12:42	12.0	MKI	Z		A	12:44	B	.	AA				A	12:54	•	.	AA			
7	04/03/90	72.7	13:14	10.0	Wet/Dry	J	••06:30	A	13:1[illegible]	F	.	A	13:15	E	14:17	CC				CC			
3	04/03/90	67.0	13:19	14.1	Syringe	J	••06:30	A	13:20	G	.	A	13:22	E	.	CC				CC			
54	04/03/90	71.0	13:24	14.2	MKI	Z		A	13:27	B	.	AA				A	13:20	E	14:30	AA			
19	04/03/90	69.5	13:50	15.0	Wet/Dry	Z		A	13:52	C	.	A	13:50	B	14:17	A	13:54	C	14:30	A	.	B	.
5	04/03/90	80.5	13:54	15.7	Syringe	J	••06:30	A	13:57	B	.	A	13:57	B	14:13	CC				CC			
33	04/03/90	71.0	14:00	15.3	MKI	Z		A	14:03	•		AA				AA				AA			
35	04/03/90	76.9	14:19	10.5	Wet/Dry	J	••06:30	A	14:26	•		A	14:22	B	14:44	CC				CC			
53	04/03/90	66.0	14:23	25.3	Wet/Dry	F	15:40	A	14:24	DD		B	14:41	DD		CC				CC			

•Sign occurred through analyzed time period.
••Animal was found dead when observer came in, in a.m.

TABLE 18.
(Continued)

Sheep I.D. Number Group #2	Dosing Date	Animal Weight (kg)	Agent Inj. Time	Actual Dose Delivered (µg/kg)	Type Treatment	Time to Death Block Code	Time to Death Noted Time	Duration of Tremors (+1 min to +120 min) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of Convulsions (+1 min to +120 min) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of Sternal/Ventral Recumbency (up to +48 hr) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of P... (up to ...) Onset Block Code	Onset Noted Time	Co...	...ed Time
20	04/18/90	80.1	12:08	11.0	Wet/Dry	Z		A	12:07	G	.	B	12:30	E	13:06	B	12:27	E	13:14	D	13:01	E	13:16
29	04/18/90	85.5	12:10	10.0	Syringe	Z		A	12:12	F	.	A	12:14	B	12:39	A	12:14	C	.	AA			
46	04/18/90	79.3	12:15	14.5	MKI	I	15:00	A	12:10	H	.	A	12:24	B	13:15	CC				CC			
22	04/18/90	77.3	12:20	12.0	Wet/Dry	Z		A	12:23	F	.	A	12:26	B	12:30	A	12:20	D	13:10	AA			
58	04/18/90	73.9	12:25	12.0	Syringe	I	15:40	A	12:20	H	.	A	12:31	G	14:02	CC				CC			
9	04/18/90	72.7	12:31	15.0	MKI	C	13:15	A	12.04	DD		A	12:34	DD		CC				CC			
57	04/18/90	85.0	12:36	14.5	Wet/Dry	D	13:36	A	.	DD		A	12:40	DD		CC				CC			
56	04/18/90	78.0	12:41	13.9	Syringe	D	14:22	A	12:44	DD		A	12:45	DD		CC				CC			
11	04/18/90	79.5	12:47	17.4	MKI	D	13:30	A	.	DD		A	12:51	DD		CC				CC			
4	04/18/90	78.5	12:55	15.0	Wet/Dry	I	16:16	A	12:58	•		A	13:01	B	13:19	CC				CC			
18	04/18/90	73.0	13:00	16.0	Syringe	Z		A	13:02	•		AA				A	13:03	C	13:45	AA			
17	04/18/90	71.1	13:05	19.5	MKI	J	••06:20	A	13:14	•		B	13:31	E	14:00	CC				CC			
12	04/18/90	74.0	13:10	17.4	Wet/Dry	Z		A	13:13	C	.	AA				A	13:14	A	13:16	AA			
2	04/18/90	80.2	13:15	19.0	Syringe	J	••06:20	A	13:19	•		AA				CC				CC			
52	04/18/90	70.5	13:20	22.0	MKI	I	17:34	A	.	•		A	13:24	C	14:02	CC				CC			
34	04/18/90	70.5	13:25	19.0	Wet/Dry	J	••06:20	A	13:27	F	.	A	13:27	A	13:36	CC				CC			
40	04/18/90	80.4	13:31	22.7	Wet/Dry	C	14:00	A	13:34	DD		A	13:36	DD		CC				CC			

•Sign continued through analyzed time period.
••Animal was found dead when observer came in, ca a.m.

Group #3																							
106	04/25/90	87.5	11:06	13.7	MKI	F	13:25	A	11:07	DD		B	11:31	DD		CC				CC			
115	04/25/90	86.0	11:12	11.7	Syringe	Z		A	.	B	.	AA				I	16:16	•		AA			
08	04/25/90	88.2	11:17	15.0	MI-6/MKI ATR	I	14:35	AA				AA				CC				CC			
144	04/25/90	81.4	11:22	14.2	MKI	Z		A	11:30	•		B	11:39	B	11:50	C	11:54	D	12:11	J	.		•
133	04/25/90	80.0	11:27	12.0	Syringe	Z		A	11:29	C	.	AA				AA				AA			
136	04/25/90	88.4	11:31	17.0	MI-6/MKI ATR	Z		A	11:40	C	.	AA				AA				AA			
125	04/25/90	80.0	11:30	15.0	MKI	J	••06:30	AA				AA				CC				CC			
40	04/25/90	87.3	11:41	14.0	Syringe	D	12:20	A	11:43	DD		A	11:44	DD		CC				CC			
132	04/25/90	85.0	11:45	19.0	MI-6/MKI ATR	I	15:27	A	11:47	•		A	11:47	G	13:30	CC				CC			
21	04/25/90	87.0	11:50	17.7	MKI	Z		A	12:04	E	.	AA				AA				AA			
45	04/25/90	75.0	11:55	16.1	Syringe	D	12:41	A	11:57	DD		A	11:59	DD		CC				CC			
30	04/25/90	80.5	11:50	20.1	MI-6/MKI ATR	Z		AA				AA				AA				AA			
14	04/25/90	79.5	12:04	18.0	MKI	H	15:55	A	12:06	DD		A	12:11	DD		CC				CC			
131	04/25/90	71.0	12:00	18.0	Syringe	Z		A	12:11	•		AA				C	12:50	F	.	AA			
119	04/25/90	82.3	12:13	22.0	MI-6/MKI ATR	I	17:29	A	12:15	•		A	12:25	C	12:49	CC				CC			

•Sign continued through analyzed time period.
••Animal was found dead when observer came in, ca a.m.

TABLE 16.
(Continued)

Sheep I.D. Number Group #4	Dosing Date	Animal Weight (kg)	Agent Inj. Time	Actual Dose Delivered (µg/kg)	Type Treatment	Time to Death Block Code	Time to Death Noted Time	Duration of Tremors (-1 min to +120 min) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of Convulsions (-1 min to +120 min) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of Sternal/Ventral Recumbency (up to +48 hr) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of P... (up to ...) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time
90	86/07/90	84.8	52	*8.7	MKI	I	15:10	A	11:50	•		A	11:54	B	12:15	CC				CC			
124	86/07/90	78	57	11.1	Syringe	Z		A	11:50	•		A	12:00	B	12:15	B	.	•		AA			
128	86/07/90	66.-	12 01	11.6	MI-C *MKI AIR	Z		A	12:53	•		A	12:11	B	12:21	AA				AA			
148	86/07/90	71.3	12 05	12.8	MKI	Z		A	12:07	•		AA				AA				AA			
94	86/07/90	78.5	12 10	12.8	Syringe	Z		A	.	E	.	AA				AA				AA			
134	86/07/90	/...	12 14	15.0	MI-6/MKI AIR	Z		A	.	•		A	12:19	B	12:29	A	.	•		AA			
168	86/07/90	72.8	12 18	12.8	MKI	J	••06:10	A	10:21	•		A	12:22	C	13:02	CC				CC			
167	86/07/90	75.5	12 22	14.0	Syringe	E	13:30	A	12:24	DD		A	12:25	DD		CC				CC			
120	86/07/90	84.8	12 30	17.1	MI-0 * ATR	I	15:40	A	12:30	G	.	A	12:32	B	12:50	CC				CC			
137	86/07/90	76.6	12 32	14.1	MKI	I	15:50	A	12:34	H	.	A	12:36	E	13:47	CC				CC			
130	86/07/90	84.6	12 30	15.1	Syringe	Z		A	12:40	H	.	A	12:48	C	13:07	B	12:52	F	.	AA			
161	86/07/90	82.8	12 48	19.0	MI-0/MKI AIR	J	••08:25	AA				AA				CC				CC			
88	86/07/90	78.5	12 44	15.1	MKI	J	••08:20	A	12:45	F	.	A	12:48	B	13:14	CC				CC			
128	86/07/90	80.8	12 48	17.0	Syringe	Z		A	12:40	•	.	AA				L	.	L	.	AA			
100	86/07/90	71.1	12 52	24.1	MI-0/MKI AIR	F	14:22	A	12:54	DD		A	12:54	DD		CC				CC			

•Sign continued through analyzed time period.
••Animal was found dead when observer came in, 10 a.m.

Group #6

122	86/30/90	72.6	10 48	9.8	MKI	Z		A	10:55	E	.	AA				AA				AA			
126	86/30/90	80.2	10 5.	9.5	Atropine	J	••08:25	A	10:56	•		AA				CC				CC			
110	85/30/90	82.3	10 55	10.1	MKI	J	••08:25	A	10:57	•		AA				CC				CC			
135	85/30/90	80.1	11 00	10.1	Atropine	Z		A	11:14	D	.	AA				AA				AA			
105	85/30/90	86.8	11 04	11.2	MKI	Z		A	11:00	•		AA				J	.	•		AA			
141	86/30/90	85.0	11 08	11.9	Atropine	I	15:00	A	11:15	H	.	AA				AA				AA			
146	85/30/90	77.7	11 12	12.0	MKI	H	13:01	A	11:27	DD		B	11:30	DD		CC				CC			
26	86/30/90	100.8	11 17	13.5	Atropine	A	11:20	A	11:22	DD		DD				CC				CC			
98	85/30/90	81.8	11 21	13.8	MKI	A	11:30	A	11:22	DD		A	11:23	DD		CC				CC			
136	85/30/90	72.8	11 25	16.5	Atropine	E	13:34	A	11:27	DD		A	11:29	DD		CC				CC			

•Sign continued through analyzed time period.
••Animal was found dead when observer came in, 10 a.m.

TABLE 18.
(Continued)

Sheep I.D. Number Group ID	Dosing Date	Animal Weight (kg)	Agent Inj. Time	Actual Dose Delivered (µg/kg)	Type Treatment	Time to Death Block Code	Time to Death Noted Time	Duration of Tremors (+1 min to +120 min) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of Convulsions (+1 min to +120 min) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of Sternal/Ventral Recumbency (up to +48 hr) Onset Block Code	Onset Noted Time	Cease Block Code	Cease Noted Time	Duration of F... (up to ...) Onset Block Code	Onset Noted Time	C...	...ted Time
102	08/13/90	73.4	10 50	7.8	Atropine	J	>+08:17	B	11:00	•		AA				CC				CC			
171	08/13/90	70.4	10 54	9.8	MKI	Z		B	11:17	B	.	AA				AA				AA			
61	08/13/90	77.3	10 58	12.5	Bot/Dry	Z		A	11:02	B	.	AA				AA				AA			
146	08/13/90	67.5	11 05	13.5	Atropine/MI-8	J	>+27:32	A	11:00	B	.	A	11:10	C	11:40	CC				CC			
90	08/13/90	66.0	11 09	8.1	Atropine	E	11:51	E	12:40	E	.	AA				CC				CC			
80	08/13/90	81.8	11 13	10.4	MKI	Z		A	11:20	E	.	B	11:40	B	11:42	A	11:20	E	12:26	AA			
97	08/13/90	70.2	11 17	14.1	Bot/Dry	Z		A	11:19	B	.	A	11:20	C	11:40	AA				A	11:20	C	12:51
160	08/13/90	67.3	11:22	14.6	Atropine/MI-8	E	12:20	A	11:24	BB		A	11:30	BB		CC				CC			
111	08/13/90	75.0	11:25	9.1	Atropine	I	14:00	B	.	•		E	12:26	•		CC				CC			
85	08/13/90	65.0	11:30	11.0	MKI	Z		A	.	B	.	AA				AA				AA			
114	08/13/90	80.4	11:35	16.1	Bot/Dry	C	12:20	A	11:20	BB		A	11:30	BB		CC				CC			
118	08/13/90	68.2	11:00	8.6	Atropine/MI-8	I	14:30	A	.	B	.	A	11:42	C	12:24	CC				CC			
10	08/13/90	90.2	11:45	11.4	Atropine	I	14:30	A	.	B	.	A	11:48	C	12:17	CC				CC			
85	08/13/90	80.0	11:50	12.5	MKI	I	14:30	AA				AA				CC				CC			
118	08/13/90	75.5	11:54	16.8	Bot/Dry	I	16:54	A	.	•		A	11:57	B	12:54	CC				CC			
144	08/13/90	75.2	11:50	21.1	Atropine/MI-8	B	12:58	A	12:00	BB		A	12:03	BB		CC				CC			
21	08/13/90	61.8	12:00	12.0	Atropine	J	>+22:30	E	19:10	B	.	AA				CC				CC			

>+ Sign continued through analyzed time period.
codeless: was found dead when observer came in.

The duration of the observation period used for incidence of tremors and convulsions was 2 hr, and 48 hr was used for sternal recumbency and prostration. Because of similarities in onset and cessation times of sternal recumbency and prostration for animals that died within 48 hr, these data were not used for analyses and are not shown in Table 10. Onset and cessation times for prostration were recorded for only four animals. All other animals either did not become prostrate or died within 48 hr. Therefore, no statistical analyses were performed on onset or duration of prostration.

Table 11 displays numbers of animals in various time categories for time to death and time to onset of tremors, convulsions, and sternal recumbency. For those animals that exhibited tremors or convulsions, the majority of onset times occurred between 0 and 15 min. Because of the large number of animals that died prior to 48 hr, the amount of data for onset of sternal recumbency was limited for each treatment group. The frequency counts did not reveal any potential treatment group effects, and therefore no further statistical analyses were done for times to onset of tremors, convulsions, or sternal recumbency.

The clinical signs selected for statistical modeling and analyses, therefore, were durations of tremors, convulsions, and sternal recumbency. When both onset and cessation times were noted, the duration in minutes was simply calculated as the cessation time minus the onset time. Clock times were not recorded for the cessation of tremors, so midpoints of the intervals in which tremors were last observed were used to calculate duration of tremors. If a clinical sign was not exhibited at all and the animal lived throughout the observation period, the duration was set equal to zero. If a clinical sign commenced and continued throughout the remainder of the observation period (animal lived), the duration was set equal to the end of the observation period minus the onset time, right-censored. This means that the actual value was not known but would have been greater than the assigned value if the observation period had been longer. If a clinical sign commenced and continued until the animal died within the observation period, the duration was set equal to the length of the observation period minus the onset time, right censored.

TABLE 11. SUMMARY OF ONSET TIMES FOR TREMORS, CONVULSIONS, AND STERNAL RECUMBENCY

Treatment Group	N	Number Survived >120 min	Number Survived >48 hrs	Time to Tremors 0-15 min	Time to Tremors 15 min-2 hrs	Tremors Not Observed in 2 hrs
Untreated	5	2	2	5	0	0
Atr/Syr	10	8	1	7	3	0
2-PAM/MKI	29	22	12	26	1	2
HI-6/Syr	24	17	10	22	1	1
HI-6/Wet-Dry	18	13	7	18	0	0
HI-6/Atropen	10	9	4	7	0	3

Treatment Group	Time to Convulsions 0-15 min	Time to Convulsions 15 min-2 hrs	Convulsions Not Observed in 2 hrs	Time to Convulsions Not Used[a]
Untreated	2	2	1	0
Atr/Syr	2	1	5	1
2-PAM/MKI	11	5	12	0
HI-6/Syr	16	0	8	0
HI-6/Wet-Dry	12	2	4	0
HI-6/Atropen	6	0	4	0

Treatment Group	Time to Sternal Recumbency 0-15 min	Time to Sternal Recumbency 15 min-48 hrs	Sternal Recumbency Not Observed in 48 hrs	Time to Sternal Recumbency Not Used[a]
Untreated	0	2	0	3
Atr/Syr	0	0	1	9
2-PAM/MKI	3	2	7	17
HI-6/Syr	2	5	3	14
Hi-6/Wet-Dry	3	1	3	11
HI-6/Atropen	1	0	3	6

[a]Values are not used due to death of animals within observation period.

Regression models were fitted to the duration data for tremors, convulsions, and sternal recumbency using duration as the response variable and ln GD dose and treatment group as the independent variables. For duration of tremors and convulsions, regression models were fitted both to the data from all the animals and to data only from animals that survived the two-hour obvservation period (2-hr survivors). For duration of sternal recumbency, the regression model was fitted to only the data from animals that survived the 48-hr observation period.

Table 12 summarizes the results for statistical models fitted to the duration of clinical signs data. For each fitted model, the estimated slope of the regression model and its standard error are displayed in the third column of the table. Slopes determined to be statistically different from zero at the 5 percent significance level are identified. A hypothesis test was conducted for each fitted model to determine if there existed any statistically significant differences between the duration times for the six groups of sheep based on the fitted model. Outcomes of the hypothesis tests are shown in the fourth column of the table. The estimated parameters of the regression models were used to predict the average duration time at a GD dose of 13.2 μg/kg (two times the estimated 48-hr GD LD_{99} of untreated sheep) for each group of sheep. The predicted group means together with 95 percent confidence intervals are shown in the last six columns of Table 12.

When models were fitted to the data from all the animals, the estimated regression slope was significantly greater than zero for both duration of tremors and convulsions. That is, duration of tremors and convulsions were predicted to increase with increasing GD dose. Also, mean durations of tremors and convulsions showed significant differences among treatment groups. Mean duration times were smallest for HI-6/Atropen, and mean duration times for untreated sheep were more than twice as long as treated groups.

When the regression model was fitted to the data from the 2-hr survivors only, the estimated slope for duration of tremors was not statistically different from zero, but was still positive. The estimated slope for duration of convulsions, however, was statistically significant when the model was fitted to data from 2-hr survivors only. Therefore, duration of tremors and convulsions were predicted to increase with increasing GD dose

TABLE 12. SUMMARY OF REGRESSION MODELING OF DURATION OF TREMORS, CONVULSIONS AND STERNAL RECUMBENCY

Clinical Sign	Animals Analyzed	Slope (SE) P-value	Group Effect	Predicted Mean Durations (Min) with 95 Percent Confidence Limits at 13.2 μg/kg GD[a]					
				Untreated	Atr/Syr	2-PAM/MKI	HI-6/Syr	HI-6/Wet-Dry	HI-6/Atropen
Duration Tremors	All	270.8[b](85.7) p = 0.0016	Yes p = 0.034	275 (168,382)	125 (75,175)	112 (83,141)	107 (76,139)	80 (43,117)	59 (9,109)
Duration Convulsions	All	232.1[b](58.6) p = 0.0001	Yes p = 0.006	167 (102,232)	53 (15,90)	40 (20,59)	40 (18,62)	31 (4,58)	1 (0,37)
Duration Tremors	2-hr Survivors only	124.4 (81.9) p = 0.129	No p = 0.505	188 (78,299)	93 (45,141)	90 (64,117)	88 (58,118)	68 (33,102)	67 (22,112)
Duration Convulsions	2-hr Survivors only	58.8[b](29.9) p = 0.049	No p = 0.638	47 (9,86)	18 (1,36)	13 (4,23)	19 (8,30)	16 (3,29)	12 (0,29)
Duration Sternal Recumbancy	48-hr Survivors only	-1907 (2147) p = 0.375	No p = 0.543	0 (0,1412)	0 (0,1796)	416 (0,1003)	773 (144,1402)	29 (0,769)	957 (0,2003)

[a] 13.2 μg/kg = 2 * 6.6 μg/kg, where 6.6 μg/kg is the 48-hr GD LD_{50} for untreated sheep as estimated by the common slopes probit model for six groups of animals.

[b] Slopes statistically different from zero at the 5 percent significance level.

when attention was restricted to 2-hr survivors. There were no statistically significant differences among average duration times of tremors or convulsions for the six groups of sheep based on the models fitted to 2-hr survivors. However, the statistical power of this test may have been reduced due to the smaller sample size of the 2-hr survivors. The relationship among treatment group means was similar to the results based on models fitted to all animals: mean duration times were shortest for HI-6/Atropen and longest for the untreated sheep.

For duration of sternal recumbency, the estimated regression slope was negative, suggesting that duration of sternal recumbency decreases with increasing GD dose. However, the large standard error associated with this negative slope shows that this conclusion may not be warranted. There were no statistically significant differences among the average duration times of sternal recumbency for the six groups of sheep based on the models fitted to the data from survivors only. The confidence intervals for the predicted group means were very wide and showed much overlap among the treatment groups. Overall, inferences for duration of sternal recumbency were extremely limited due to the small amount of data.

Results displayed in Table 12 indicate similar conclusions were obtained from modeling the data from all the animals and modeling the data from the 2-hr survivors only. These results may be summarized as follows:

(1) Durations of tremors or convulsions were positively related to GD dose, with durations predicted to increase with higher GD doses. This relationship was statistically significant for durations of tremors and convulsions for all animals, and for duration of convulsions for 2-hr survivors.

(2) Analyses of durations of tremors and convulsions from all the animals revealed significant treatment group effects. Mean durations were shortest for HI-6/Atropen, and longest for the untreated sheep. Though not statistically significant, this same relationship was observed among the group means when the analyses were restricted to the 2-hr survivors.

(3) Duration of sternal recumbency among 48-hr survivors did not show any significant relationship with either GD dose or treatment group. Inferences from these analyses were limited due to the reduced sample size.

3.3.2 Pathology

Necropsies were performed on 60 sheep. These necropsies were conducted as soon after death as possible. No tissues from any animals in this study were preserved for histologic examination. Gross observations are tabulated in Table 1 and summarized in Table 2 of Appendix C. Treatment-related lesions consisted of mild to marked congestion and/or hemorrhage of the lungs, heart, thymus, trachea, and small intestine. These lesions are compatible with GD intoxication, and all deaths were directly attributed to agent toxicity. Other minor gross lesions were noted, as indicated in the tables, but these were incidental to the GD administration, and were principally developmental changes or due to parasitism.

3.4 Pharmacokinetic Study

Two IM delivery systems for HI-6 and atropine, namely three hypodermic syringes or three wet/dry autoinjectors per sheep, were used to deliver doses of 1380 mg of HI-6 and 5.6 mg of atropine sulfate equivalents to each of eight sheep in a cross-over study. A crossover design balanced for delivery system effects, test week effects, and residual effects was used in the experiment and is presented in Table 13. Blood samples were collected prior to testing and at 1, 2, 3, 4, 5, 6, 8, 12, 16, 20, 40, 60, 80, 120, 180, 240, 300, and 360 min after injection. Plasma HI-6 and serum atropine concentrations measured for each animal at each time of sampling are presented in Appendix D.

On the first day of pharmacokinetic studies, several wet/dry autoinjectors failed to inject their contents, and sheep 92 received the contents of only two autoinjectors. These injector failures appeared to be due to a technician error because the autoinjectors did express their contents

TABLE 13. TREATMENT SCHEDULE FOR PHARMACOKINETIC STUDIES

Animal ID	Week 1 of Dosing[a]	Week 2 of Dosing
23	Syringe	Wet/Dry
25	Syringe	Wet/Dry
39	Syringe	Wet/Dry
86	Wet/Dry	Syringe
92[b]	Wet/Dry	Syringe
112	Wet/Dry	Syringe
140	Wet/Dry	Syringe
142	Syringe	Wet/Dry

[a] Within week 1, animals 25, 140, 142 were dosed on 6/25/90; animals 23, 39, 86 and 112 were dosed on 6/26/90. Within week 2, animals 25, 92, 140, and 142 were dosed on 7/3/90; animals 23, 39, 86, and 112 were dosed on 7/5/90.

[b] Animal 92's treatment of 6/25/90 was repeated on 8/15/90. For statistical analysis purposes, the week factor for the wet/dry results was treated as week 1.

when the barrels were rotated at a later time. One technician was relatively inexperienced with the wet/dry autoinjectors and may not have rotated the barrels sufficient prior to attempted injection. Sheep 92 was reinjected at a later date with three wet/dry autoinjectors and blood samples obtained.

For each animal and delivery method, AUC_{0-360}, C_{max}, and t_{max} were calculated from the empirical data. Area under the blood concentration-time curve between 0 and 360 minutes was computed by the trapezoidal method. C_{max} was presumed to be the maximum observed concentration, and t_{max} the time of sampling corresponding to the maximum concentration. In addition, for each combination of animal and delivery technique, HI-6 and atropine blood concentrations were modeled as functions of time, using appropriate compartmental pharmacokinetic models. To determine the type of models which best fit the data and to estimate initial or "seed" values for parameters of that model, mean concentration values as a function of time for all sheep with both delivery methods were graphed using a personal computer. Figures 4 and 5 are graphs of modeled mean data for HI-6 and atropine, respectively. A one-compartment model provided the best fit for the HI-6 mean data, and a two-compartment model best fit the atropine mean data. Pharmacokinetic parameters estimated for HI-6 were V_d, k_e and k_{el} in the following single-compartment model:

$$C_{(t)} = \frac{D}{V_d} \frac{k_a}{(k_a - k_{el})} (e^{-k_{el}t} - e^{-k_a t})$$

where $C_{(t)}$ is the HI-6 blood concentration at time t after dosing, and D is the dose of HI-6 delivered.

Pharmacokinetic parameters estimated for atropine were A, B, α, β, and k_e in the following two-compartment model:

$$C_{(t)} = A(e^{-\alpha t} - e^{-k_e t}) + B(e^{-\beta t} - e^{-k_e t}).$$

The blood concentration over time data, for each animal and both injection techniques, were transferred to Battelle's VAX mainframe computer for more precise estimation of the parameters of one- and two-compartment models for

FIGURE 4. MEAN PLASMA HI-6 CONCENTRATIONS FOLLOWING INJECTION OF EIGHT SHEEP USING TWO DIFFERENT TECHNIQUES

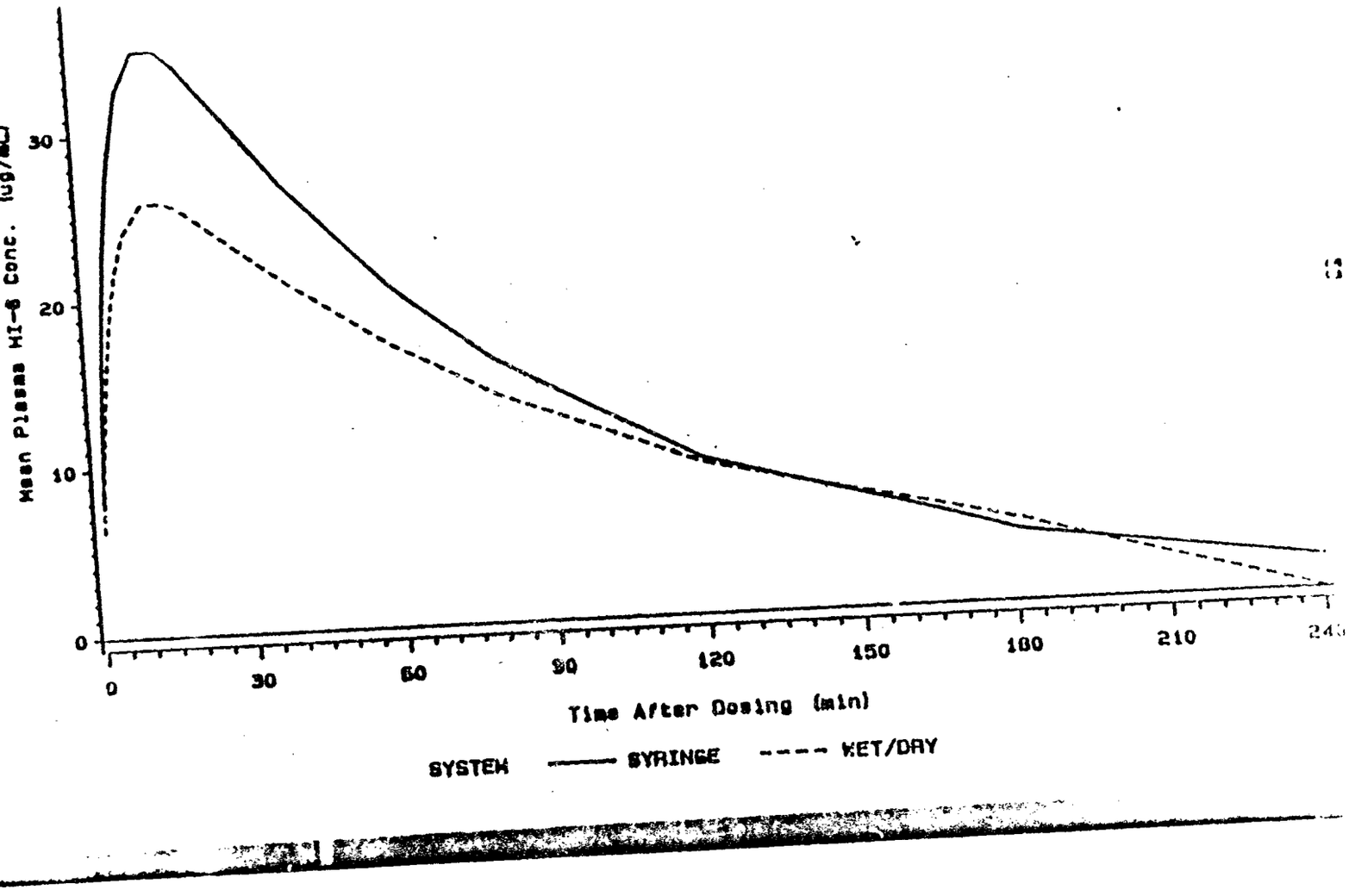
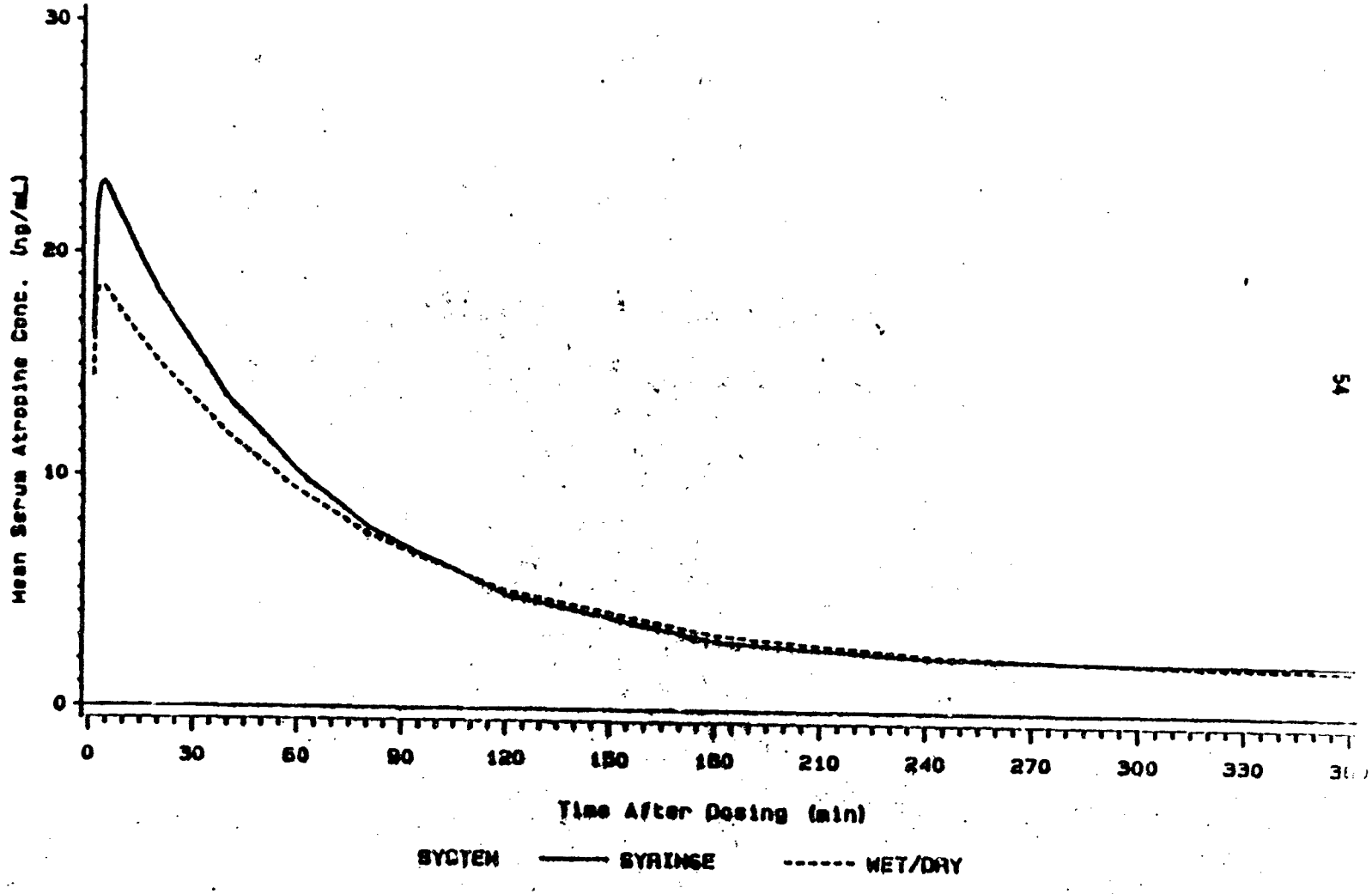


FIGURE 5. MEAN SERUM ATROPINE CONCENTRATIONS FOLLOWING INJECTION OF EIGHT SHEEP USING TWO DIFFERENT TECHNIQUES



HI-6 and atropine data, respectively. Parameter estimations were performed on the VAX using the Statistical Analysis System (SAS; Cary, NC) NLIN (nonlinear regression) program. Additional pharmacokinetic parameters were then calculated from the estimated parameters of the models and statistically analyzed. The set of pharmacokinetic parameters statistically analyzed are:

k_a - first-order rate constant for appearance of a drug in the systemic circulation (min^{-1}),

k_{el} - first-order rate constant for drug elimination by all routes (min^{-1}),

AUC_{0-360} - area under the drug concentration versus time curve from t = zero to t = 360 min (μg of HI-6 * min/mL, or ng of atropine * min/mL),

C_{max} - peak or maximum concentration (μg of HI-6/mL, or ng of atropine/mL),

t_{max} - time to maximum concentration (min),

V_d - apparent volume of distribution of a drug that obeys single-compartment kinetics, computed only for HI-6, (L),

V_{dss} - overall apparent volume of distribution of a drug that obeys two-compartment kinetics, as calculated by the area method, computed only for atropine, (L),

- $V_1(k_{el}/\beta)$ where $V_1 = D/A+B$.

Pharmacokinetic parameters, as determined by modeling data for atropine and HI-6 for each animal, are contained in Appendix D.

There were no observable signs of atropinization in sheep used in pharmacokinetic studies. Pupillary size and response to light at 20 min after injection of atropine and HI-6 did not appear to be different than that observed prior to injection. Pupillary response to light was slow and limited at both time points. There was virtually no difference in heart rates at the two observation times. Muscle coordination and ambulation at 120 min after injection, when sheep were removed from restraint, did not appear different than that observed prior to restraint.

3.4.1 Statistical Analyses of HI-6 Pharmacokinetic Parameters

Pharmacokinetic parameters were statistically analyzed to determine if there were any effects due to type of delivery or week of testing, and to assess the variability in the pharmacokinetic parameters among the animals. Empirically derived values of the HI-6 pharmacokinetic parameters $AUC_{0\text{-}360}$, C_{max}, and t_{max} are presented in Table 14. HI-6 pharmacokinetic parameters calculated from single-compartment models are shown in Tables 15 and 16. Model-based estimates of $AUC_{0\text{-}360}$, C_{max}, and t_{max} are plotted against the empirically determined values in Figures 6, 7, and 8. The plots demonstrate that there exists a strong linear relationship between the model-based and empirically determined values of $AUC_{0\text{-}360}$ and C_{max}. Correlations were computed between the empirically and model-based values, and correlations were determined to be statistically different (at the 5 percent significance level) from zero for all three parameters. Correlations between the two sets of estimates are:

Parameter	n	Correlation	P-value
$AUC_{0\text{-}360}$	16	0.982	0.0001
C_{max}	16	0.954	0.0001
t_{max}	16	0.758	0.0007

Table 17 summarizes the results of statistical analyses and hypothesis testing for delivery technique, animal-to-animal, and week of testing variability. The average values of the pharmacokinetic parameters estimated for both delivery techniques are shown in the second and third columns of the table. Because the experiments were balanced for delivery technique, the standard errors of the averages are identical for both of the techniques. The standard error of the average pharmacokinetic parameter for both injection methods is displayed in the fourth column of the table. For each HI-6 pharmacokinetic parameter, a statistical hypothesis test was performed to determine if the effect of delivery was statistically significant. The values of the F tests and their observed significance levels are given in the next two columns of the table.

TABLE 14. HI-6 PHARMACOKINETIC PARAMETERS AUC_{0-360}, C_{max}, and t_{max} DERIVED[a] FROM EMPIRICAL DATA

Animal	Test Week	Delivery System	AUC_{0-360} (μg-min/mL)	C_{max} (μg/mL)	t_{max} (min)
23	1	Syringe	3,929	63.2	20.3
	2	Wet/Dry	3,637	49.8	40.0
25	1	Syringe	2,106	23.3	40.5
	2	Wet/Dry	1,234	16.2	12.0
39	1	Syringe	2,906	42.9	12.0
	2	Wet/Dry	1,840	19.3	20.0
86	2	Syringe	2,452	30.6	12.0
	1	Wet/Dry	2,579	32.9	16.0
92	2	Syringe	2,536	28.5	20.0
	1	Wet/Dry	3,072	35.4	20.0
112	2	Syringe	2,434	25.6	40.0
	1	Wet/Dry	3,081	42.0	20.5
140	2	Syringe	3,453	51.0	20.0
	1	Wet/Dry	917	8.8	40.0
142	1	Syringe	3,155	36.5	16.0
	2	Wet/Dry	2,678	31.9	16.7

[a] AUC_{0-360} was calculated from the observed HI-6 plasma concentration-time curve using the trapezoidal method, C_{max} is the maximum observed concentration, and t_{max} is the sampling time corresponding to the maximum observed concentration.

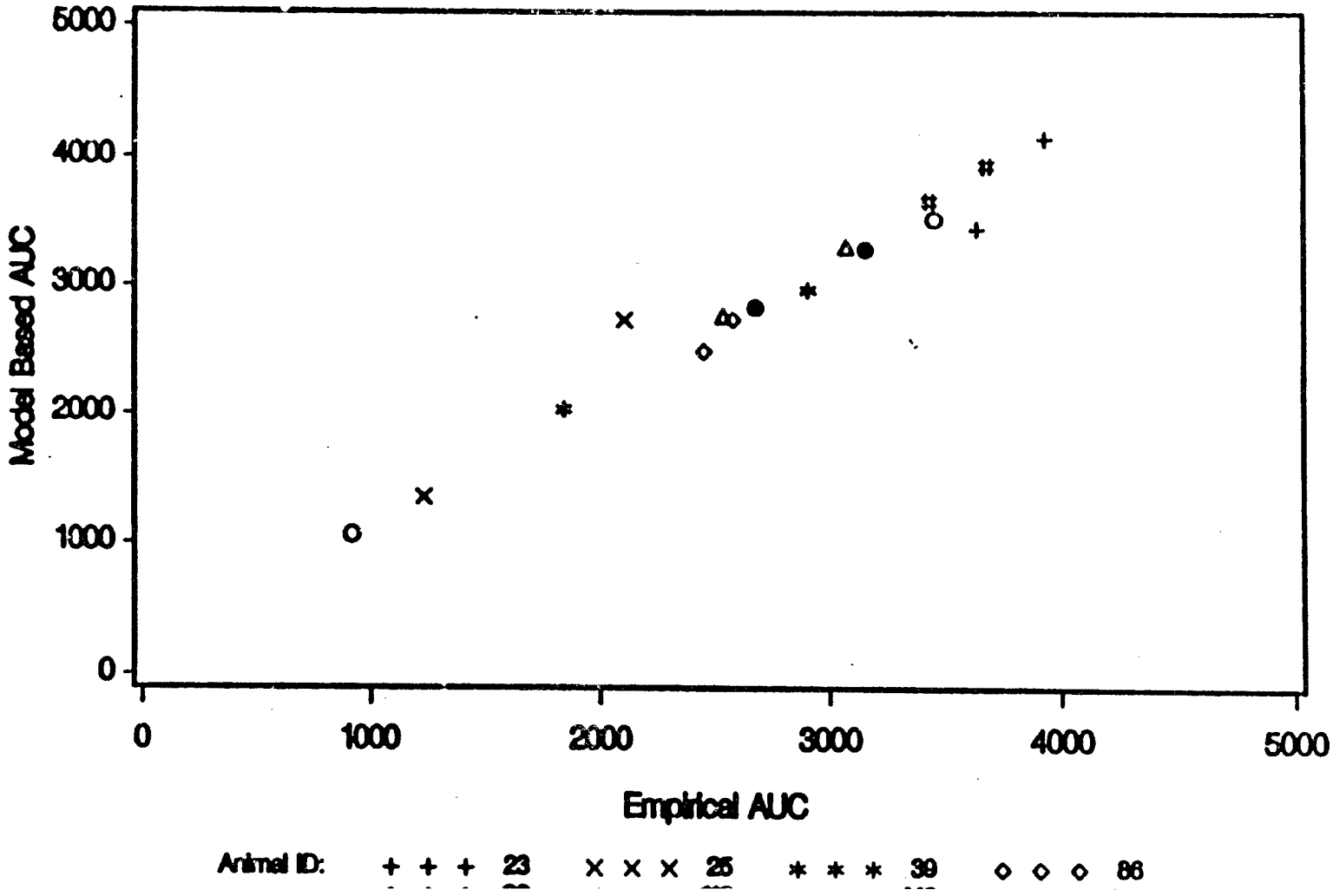
TABLE 15. HI-6 PHARMACOKINETIC PARAMETERS V_d, k_e, k_{el} FROM ONE-COMPARTMENT MODEL

Animal	Delivery Technique	Test Week	k_e (min^{-1})	k_{el} (min^{-1})	V_d (L)
23	Syringe	1	0.371	0.015	22.13
	Wet/Dry	2	0.052	0.019	21.14
25	Syringe	1	0.158	0.010	49.28
	Wet/Dry	2	0.112	0.017	60.14
39	Syringe	1	0.335	0.017	27.44
	Wet/Dry	2	0.222	0.011	62.06
85	Syringe	2	0.146	0.016	34.77
	Wet/Dry	1	0.261	0.014	36.36
92	Syringe	2	0.173	0.012	39.67
	Wet/Dry	1	0.201	0.013	32.33
112	Syringe	2	0.080	0.009	40.10
	Wet/Dry	1	0.150	0.013	26.15
140	Syringe	2	0.197	0.016	23.76
	Wet/Dry	1	0.026	0.020	65.36
142	Syringe	1	0.149	0.015	28.51
	Wet/Dry	2	0.247	0.013	37.62

TABLE 16. HI-6 PHARMACOKINETIC PARAMETERS CALCULATED FROM k_o, k_{el}, AND V_d BASED ON ONE-COMPARTMENT MODEL

Animal	Test Week	Delivery System	$AUC_{0-\infty}$ (μg·min/mL)	C_{max} (μg/mL)	t_{max} (min)
23	1	Syringe	4,154	54.6	9.0
	2	Wet/Dry	3,444	36.7	30.4
25	1	Syringe	2,727	23.3	18.7
	2	Wet/Dry	1,350	16.4	19.8
39	1	Syringe	2,963	42.9	9 4
	2	Wet/Dry	2,030	19.1	14.4
86	2	Syringe	2,483	30.2	17.0
	1	Wet/Dry	2,730	32.2	11.9
92	2	Syringe	2,761	28.3	16.4
	1	Wet/Dry	3,302	35.3	14.6
112	2	Syringe	3,667	26.1	30.8
	1	Wet/Dry	3,944	41.7	17.7
140	2	Syringe	3,523	46.3	13.8
	1	Wet/Dry	1,062	8.9	43.7
142	1	Syringe	3,280	37.5	17.3
	2	Wet/Dry	2,828	31.1	12.6

FIGURE 6. MODEL-BASED VERSUS EMPIRICAL AUC_{0-360} FOR PLASMA HI-6



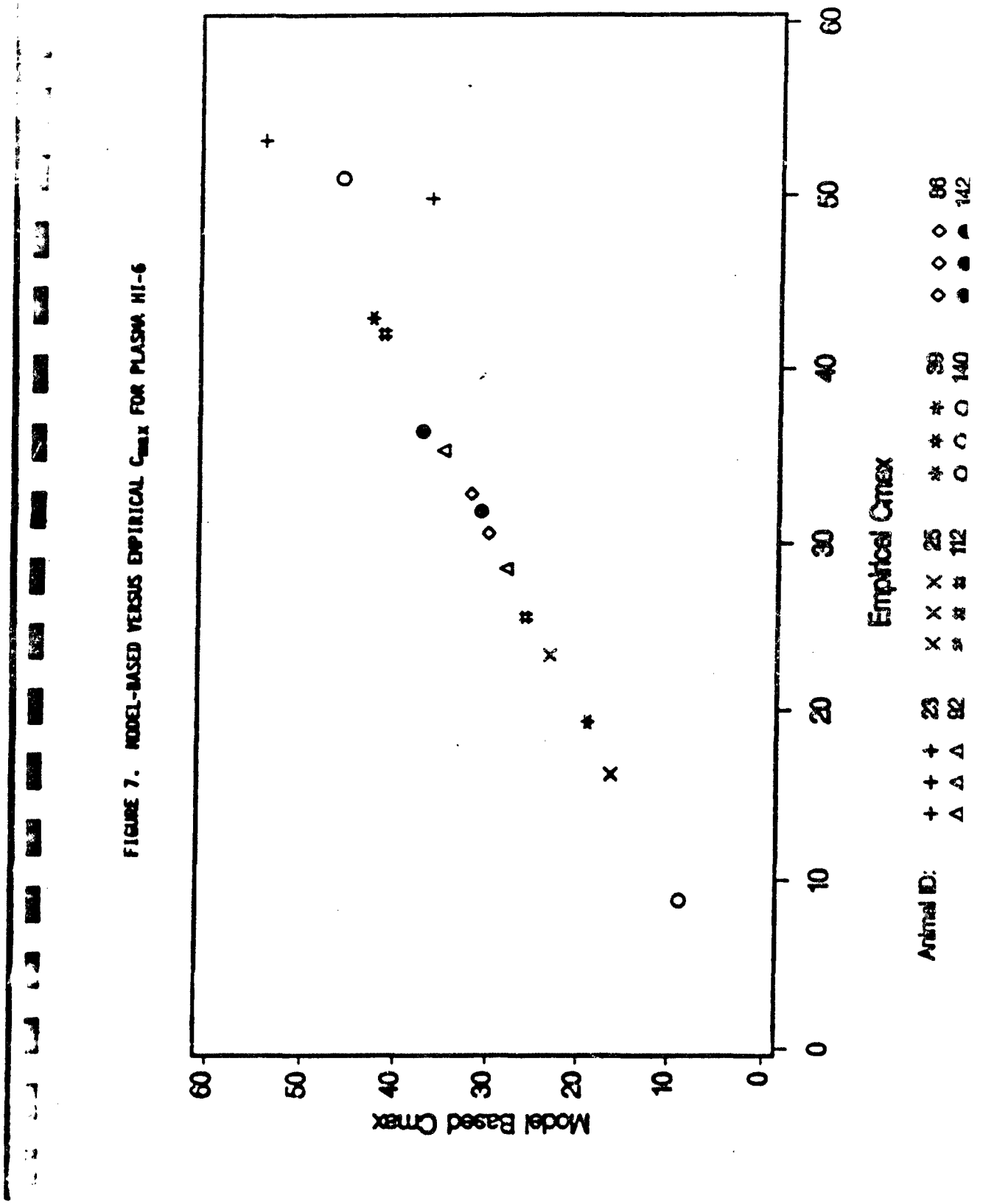


FIGURE 7. MODEL-BASED VERSUS EMPIRICAL C_{max} FOR PLASMA HI-6

FIGURE 8. MODEL-BASED VERSUS EMPIRICAL t_{max} FOR PLASMA HI-6

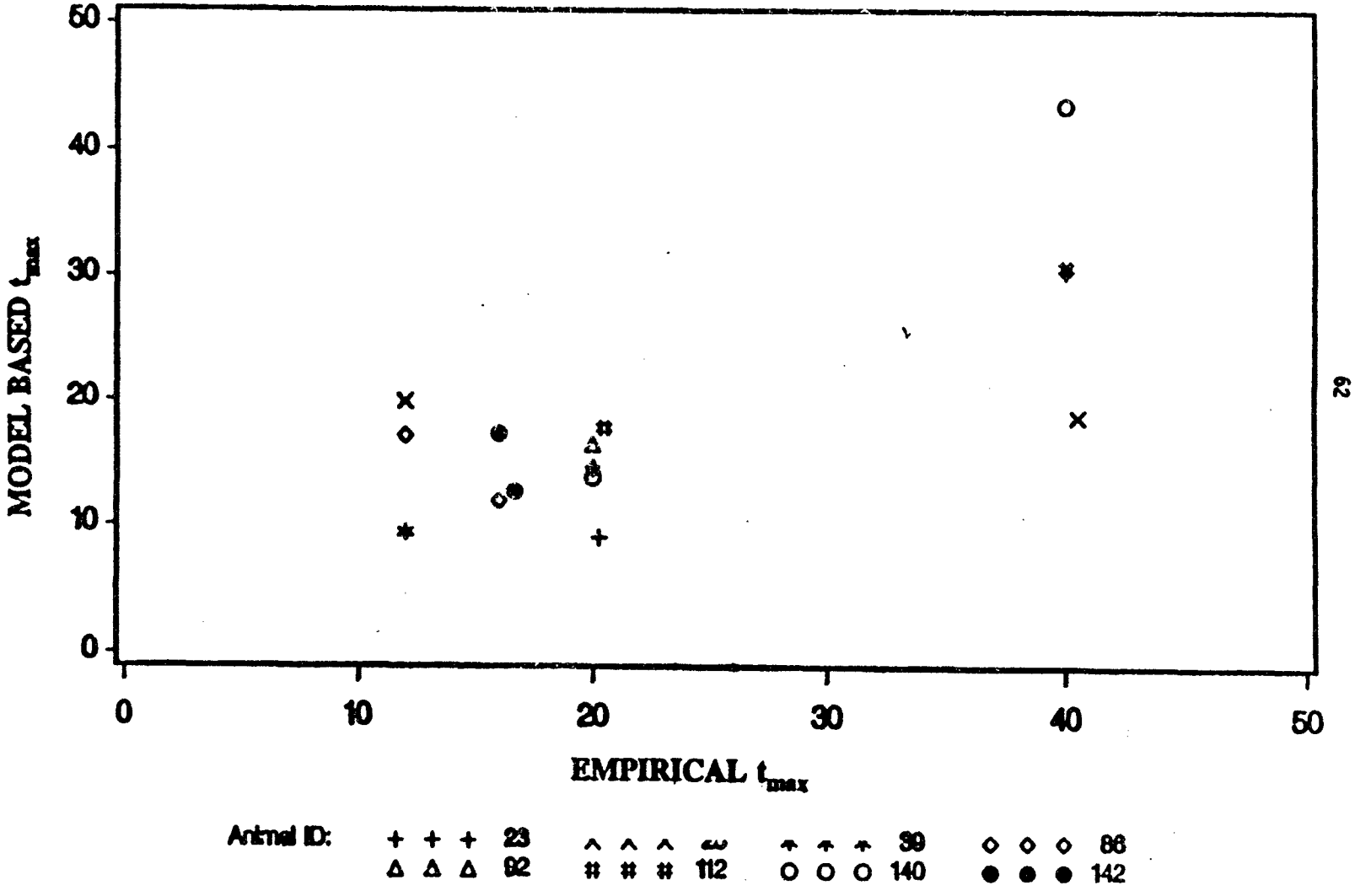


TABLE 17. SUMMARY OF STATISTICAL ANALYSIS OF DELIVERY SYSTEM, ANIMAL-TO-ANIMAL, AND WEEK OF TESTING VARIABILITY FOR HI-6 PHARMACOKINETIC PARAMETERS

Pharmacokinetic Parameter (units)	Effect of Delivery System: Model Predicted Average Wet/Dry	Model Predicted Average Syringe	SE[a] of Average	F-Value	P-Value[b]	Animal Variability[c]: σ^2_A	σ^2_A/σ^2_e	P-Value	Week of Dosing: F-Value	P-Value
Empirical Parameters										
AUC_{0-360} (μg•min/mL)	2,455	2,996	261	2.16	0.19	218,416	0.402	0.24	0.13	0.73
C_{max} (μg/mL)	29.5	36.5	4.9	1.00	0.36	0.000	0.000	0.62	0.16	0.70
t_{max} (min)	23.2	22.6	4.6	0.01	0.94	0.000	0.000	0.76	0.01	0.93
Model-Based Parameters										
k_e (min^{-1})	0.159	0.201	0.038	0.62	0.46	0.000	0.000	0.77	0.98	0.36
k_{el} (min^{-1})	0.015	0.014	0.001	0.45	0.53	0.000	0.000	0.53	0.05	0.83
AUC_{0-360} (μg•min/mL)	2,586	3,195	259	2.75	0.15	163,455	0.304	0.29	0.50	0.51
C_{max} (μg/mL)	27.7	36.2	4.4	1.84	0.22	0.000	0.000	0.70	0.71	0.43
t_{max} (min)	20.7	16.5	3.9	0.56	0.48	0.000	0.000	0.79	0.09	0.78
V_d(L)	42.6	33.2	5.2	1.68	0.24	0.000	0.000	0.54	0.30	0.61

[a] Standard error of the estimated average value of the pharmacokinetic parameter for each delivery system. Because the experimental design was balanced across delivery technique, the standard errors are the same for both delivery systems.

[b] Observed significance level.

[c] σ^2_A - Estimate of the animal-to-animal variance component.

σ^2_A/σ^2_e - Ratio of the variance components estimated for animals to the variance component estimated for uncontrolled error.

P-value - Observed significance level for the animal-to-animal variance component.

The component of variation due to the effects of the different animals was estimated for each HI-6 pharmacokinetic parameter. The estimates of the between animal variance components (σ_A^2) are displayed in column seven of Table 17. Negative estimates of the variances were reported as zero. To assess the magnitude of animal-to-animal variability, the between animal variance components were statistically compared to the variance component estimated for the variability within animals (σ^2). Ratios of the two variance components, and statistical significance levels for the between animal variance component are displayed in the eight and ninth columns of the table. For each pharmacokinetic parameter, a statistical hypothesis test was performed to determine if the effect of week was statistically significant. The values of the F tests and their observed significance levels are displayed in the last two columns of the table.

Effects of delivery technique, animal variability, and test week were determined to be statistically insignificant (at the 5 percent level) for all of the nine HI-6 pharmacokinetic parameters. Numerically, the mean values of the empirical and model-based estimates of $AUC_{0-\infty}$ and C_{max} were somewhat greater for syringe delivery than for the wet/dry autoinjector.

2.4.2 Statistical Analyses of Atropine Pharmacokinetic Parameters

Empirical values of the atropine pharmacokinetic parameters $AUC_{0-\infty}$, C_{max}, and t_{max} are presented in Table 18. Atropine pharmacokinetic parameters calculated from two-compartment models are shown in Tables 19 and 20. The absorption rate constant, (k_a), was not able to be determined from the injection by syringe data for animal 39. This was the result of very rapid absorption of the atropine when delivered by syringe to this animal. The model-based estimated k_a value was so large that it was essentially unquantifiable. This problem did not appear to adversely affect, however, the ability of the pharmacokinetic model to estimate the remaining parameters for this animal.

Model-based estimates of $AUC_{0-\infty}$, C_{max}, and t_{max} are plotted against the empirically determined values in Figures 9, 10, and 11. The plots demonstrate that a strong linear relationship exists between the model-based

TABLE 18. ATROPINE PHARMACOKINETIC PARAMETERS AUC_{0-360}, C_{max}, AND t_{max} DERIVED[a] FROM EMPIRICAL DATA

Animal	Test Week	Delivery System	AUC_{0-360} (ng·min/mL)	C_{max} (ng/mL)	t_{max} (min)
23	1	Syringe	2,304	37.9	2.0
	2	Wet/Dry	2,191	27.4	3.0
25	1	Syringe	2,036	18.4	16.0
	2	Wet/Dry	1,574	13.5	12.0
39	1	Syringe	1,843	34.9	2.2
	2	Wet/Dry	1,533	19.6	3.0
86	2	Syringe	1,768	24.0	12.0
	1	Wet/Dry	1,937	29.5	2.0
92	2	Syringe	2,258	25.0	3.0
	1	Wet/Dry	1,848	19.8	3.0
112	2	Syringe	2,029	17.2	20.0
	1	Wet/Dry	2,084	31.0	3.0
140	2	Syringe	1,839	33.1	2.0
	1	Wet/Dry	1,063	11.8	20.0
142	1	Syringe	2,621	31.3	12.0
	2	Wet/Dry	2,246	26.6	3.3

[a] AUC_{0-360} was calculated from the serum atropine concentration-time curve using the trapezoidal method, C_{max} is the maximum observed concentration, and t_{max} is the sampling time corresponding to the maximum observed concentration.

TABLE 19. ATROPINE PHARMACOKINETIC PARAMETERS A, B, α, β AND k_o FROM TWO-COMPARTMENT MODEL

Animal	Delivery System	Test Week	A	B	α	β	k_o (min^{-1})
23	Syringe	1	32.03	4.33	0.025	0.003	1.644
	Wet/Dry	2	274.50	13.29	0.413	0.006	0.475
25	Syringe	1	15.12	1.59	0.008	0.004	1.133
	Wet/Dry	2	11.96	3.51	0.015	0.003	0.298
39	Syringe	1	26.51	6.44	0.043	0.004	[a]
	Wet/Dry	2	15.64	3.36	0.022	0.002	1.048
86	Syringe	2	19.58	2.99	0.019	0.002	1.672
	Wet/Dry	1	26.00	3.76	0.027	0.002	1.941
92	Syringe	2	20.24	4.24	0.016	0.003	0.957
	Wet/Dry	1	14.83	4.44	0.016	0.004	2.311
112	Syringe	2	11.70	5.36	0.011	0.004	0.335
	Wet/Dry	1	20.17	4.24	0.018	0.003	1.031
140	Syringe	2	28.37	4.40	0.035	0.003	1.226
	Wet/Dry	1	-60.40	75.85	0.005	0.005	0.095
142	Syringe	1	143.30	14.05	0.081	0.006	0.120
	Wet/Dry	2	23.13	4.69	0.019	0.003	2.753

[a] It was not possible to adequately estimate k_o from the data collected due to rapid absorption of atropine.

TABLE 20. ATROPINE PHARMACOKINETIC PARAMETERS CALCULATED FROM A, B, α, β, AND k_a BASED ON TWO-COMPARTMENT MODEL

Animal	Test Week	Delivery System	k_{el} (min^{-1})	$AUC_{0-\infty}$ (ng·min/mL)	C_{max} (ng/mL)	t_{max} (min)	V_d (L)
23	1	Syringe	0.013	2,214	33.7	3.0	709
	2	Wet/Dry	0.020	2,094	17.8	6.9	70
25	1	Syringe	0.008	2,041	16.0	4.5	590
	2	Wet/Dry	0.007	1,561	12.9	12.9	992
39	1	Syringe	0.014	1,851	32.9	0.0	638
	2	Wet/Dry	0.008	1,532	17.3	4.7	1159
86	2	Syringe	0.010	1,712	21.3	3.1	1038
	1	Wet/Dry	0.011	1,893	27.7	2.7	973
92	2	Syringe	0.009	2,198	22.7	5.0	720
	1	Wet/Dry	0.009	1,810	18.7	2.4	706
112	2	Syringe	0.007	2,010	15.0	11.8	594
	1	Wet/Dry	0.010	2,041	22.6	4.6	713
140	2	Syringe	0.013	1,811	28.9	3.8	855
	1	Wet/Dry	0.007	1,991	11.9	29.7	502
142	1	Syringe	0.021	2,590	28.4	17.6	129
	2	Wet/Dry	0.010	2,193	26.8	2.0	660

FIGURE 9. MODEL-BASED VERSUS EMPIRICAL AUC_{0-360} FOR SERUM ATROPINE

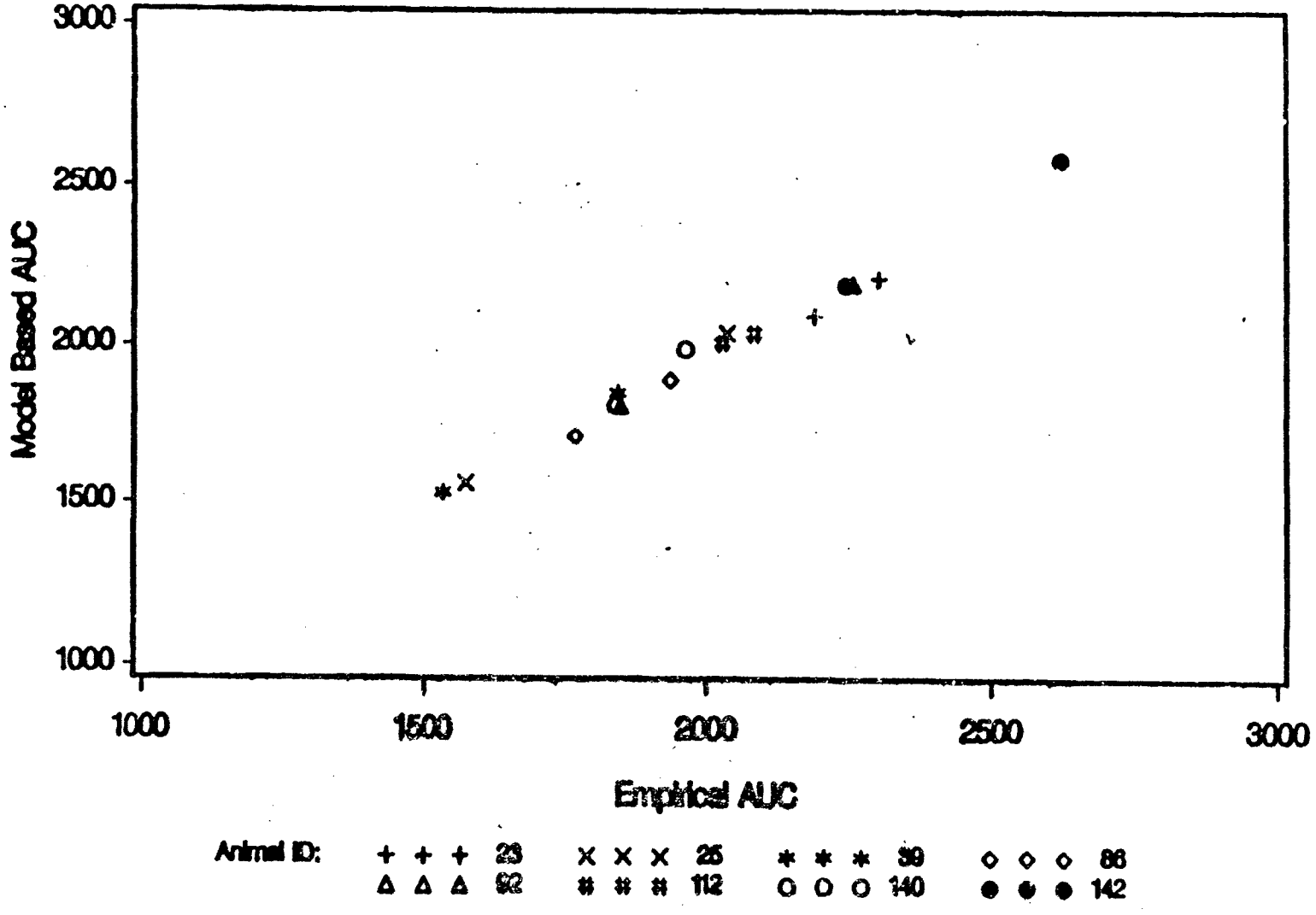


FIGURE 10. MODEL-BASED VERSUS EMPIRICAL C_{max} FOR SERUM ATROPINE

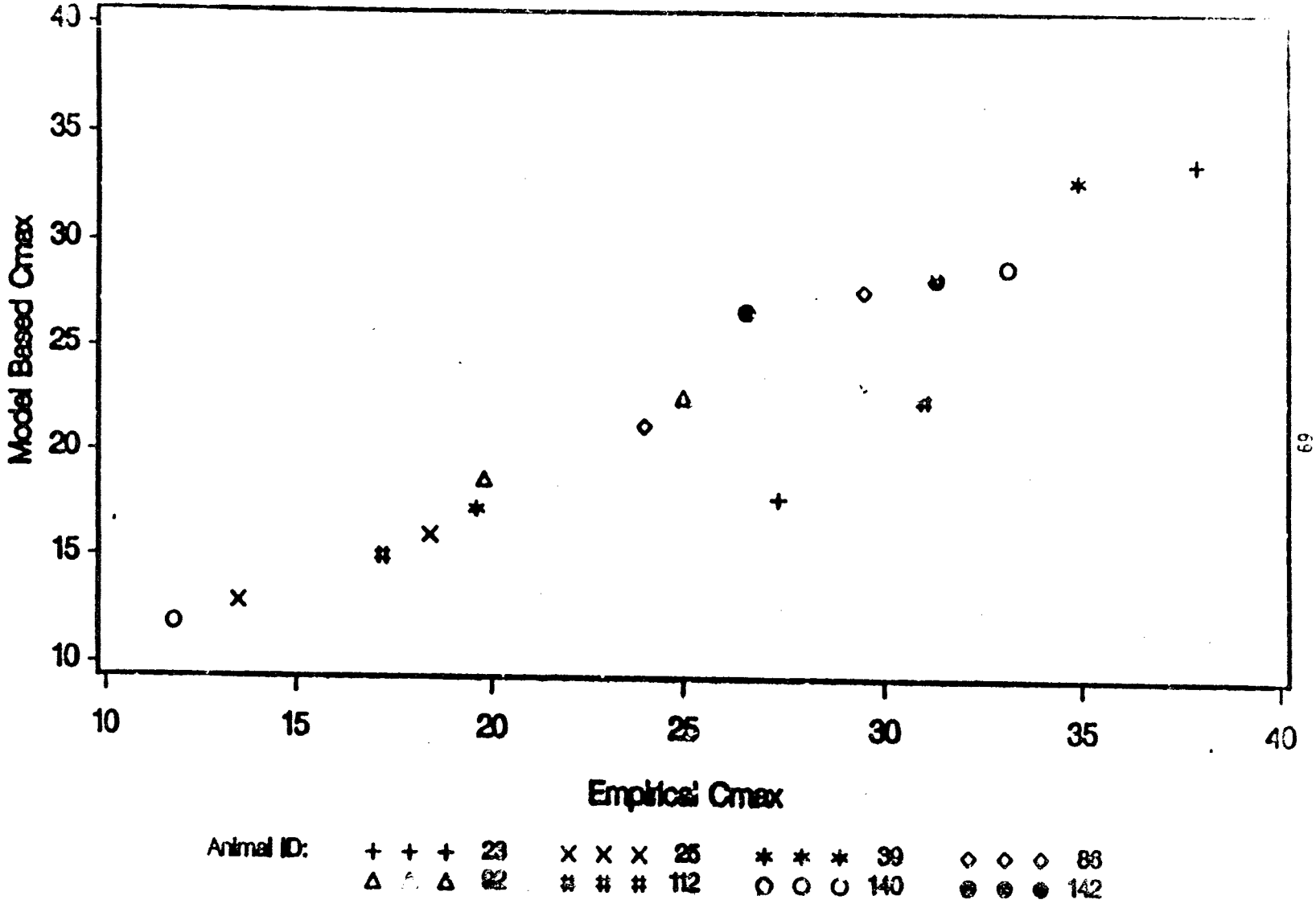
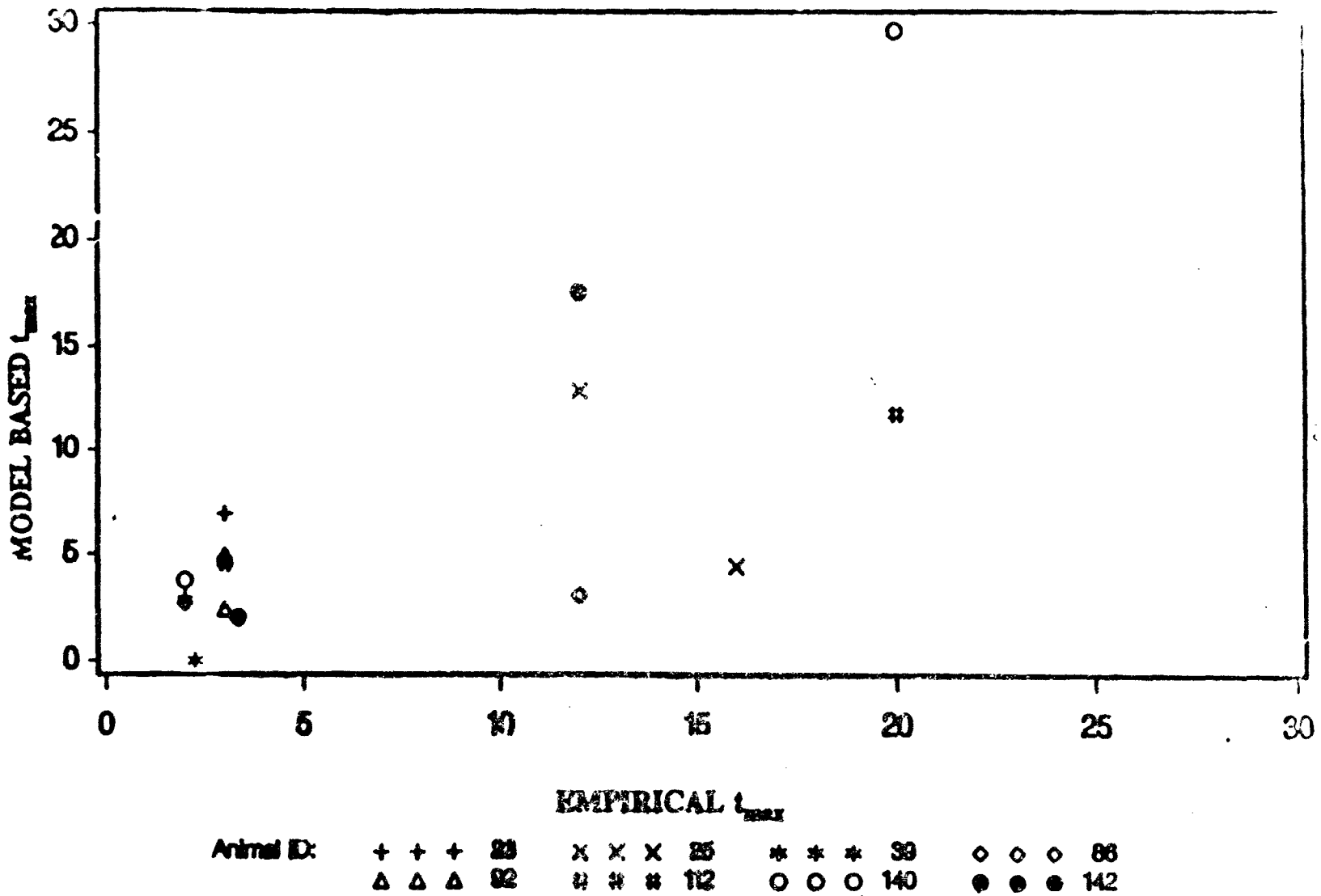


FIGURE 11. MODEL-BASED VERSUS EMPIRICAL t_{max} FOR SERUM ATROPINE



and empirically determined values of AUC_{0-360} and C_{max}. Correlations were computed between the empirical and model-based values of these parameters, and correlations were determined to be statistically different (at the 5 percent significance level) from zero for all three parameters. Correlations calculated between the two sets of estimates are:

Parameter	n	Correlation	P-value
AUC_{0-360}	16	0.994	0.0001
C_{max}	16	0.938	0.0001
t_{max}	16	0.719	0.0017

Statistical procedures utilized to analyze the atropine pharmacokinetic parameters are analogous to those employed to analyze the HI-6 estimates for all parameters except k_e. The unquantifiable k_e value for sheep 39 was treated as right-censored at 3.0 min^{-1}. This means that the k_e value is not known but would have been greater than or equal to the assigned value if it had been able to be estimated. This approach allowed an analysis of variance on 16 k_e values, one of which was treated as right-censored. The presence of right-censored data, however, required specialized programs employing maximum likelihood methods rather than least square techniques to perform the analysis of variance. Therefore, test statistics assessing the significance of effects on the k_e parameter have an approximate chi-square distribution instead of the F distribution employed for the other parameters.

Hypothesis tests were conducted for each pharmacokinetic parameter to determine the statistical significance of effects of delivery technique, animal, and week of testing. Table 21 summarizes the results of statistical analysis and hypothesis testing for injection technique, animal-to-animal, and week of testing variability. While the effects of delivery were determined to be statistically insignificant (at the 5 percent level) for all of the pharmacokinetic parameters, they were marginally significant for the empirical and model-based estimates of AUC_{0-360} (P=0.07 and P=0.08, respectively). Numerically, the mean values of the empirical and model-based estimates of AUC_{0-360} and C_{max} were somewhat greater for syringe delivery than for the wet dry autoinjector. The between animal variance component was determined to be statistically significant for the empirical and model-based estimates of

TABLE 21. SUMMARY OF STATISTICAL ANALYSIS OF DELIVERY SYSTEM, ANIMAL-TO-ANIMAL, AND WEEK OF TESTING VARIABILITY FOR ATROPINE PHARMACOKINETIC PARAMETERS

Pharmacokinetic Parameter (units)	Effect of Delivery System					Animal Variability[c]			Week of Dosing	
	Model Predicted Avg. Wet/Dry	Model Predicted Avg. Syringe	SE[a] of Avg.	F-Value	P-Value[b]	σ^2_A	σ^2_A/σ^2_e	P-Value	F-Value	P-Value
Empirical Parameters										
$AUC_{0-\infty}$ (ng·min/mL)	1922	2087	54	4.67	0.07	48621	2.079	0.03	3.84	0.10
C_{max} (ng/mL)	22.4	27.7	2.8	1.78	0.23	0.000	0.000	0.62	0.78	0.41
t_{max} (min)	6.2	8.7	2.8	0.39	0.55	0.000	0.000	0.72	0.00	0.95
Model-Based Parameters										
k_a (min^{-1})[d]	0.991	1.214	[e]	0.20	0.66			0.34	0.05	0.82
k_{el} (min^{-1})	0.010	0.012	0.002	0.48	0.51	0.000	0.113	0.41	0.19	0.68
$AUC_{0-\infty}$ (ng·min/mL)	1889	2053	55	4.47	0.08	38721	1.613	0.05	4.52	0.08
C_{max} (ng/mL)	19.5	24.9	2.4	2.45	0.17	0.000	0.000	0.62	1.12	0.33
t_{max} (min)	8.2	6.1	3.3	0.22	0.66	0.000	0.000	0.78	0.15	0.71
V_{dp} (L)	722	659	106	0.18	0.69	2740.6	0.031	0.48	0.89	0.38

(a) Standard error of the estimated average value of the pharmacokinetic parameter for both delivery systems. Because the experimental design was balanced across delivery systems, the standard errors are the same for both delivery techniques.

(b) P-value = Observed significance level.

(c) σ^2_A = Estimate of the animal-to-animal variance component.

σ^2_A/σ^2_e = Ratio of the variance components estimated for animals to the variance component estimated for uncontrolled error.

(d) Because it was not possible to estimate k_a for one animal, log-likelihood procedures were used to statistically analyze the k_a data. Therefore, test statistics follow a chi-square rather than a F-distribution.

(e) Standard errors are 0.287 and 0.285 for the wet/dry and syringe delivery systems, respectively.

AUC_{0-360}. The variation in pharmacokinetic parameters over the two weeks of testing was determined to be statistically insignificant for all parameters.

3.5 GD Aging

GD inhibition of ovine RBC and eel AChE activity data are presented in Figure 12. The level of AChE activity in samples from ten different sheep and from a single eel AChE preparation was examined at GD concentrations ranging from 0.10 nM to 8.33 nM. The average GD concentration which produced 50 percent inhibition of sRBC AChE (IC_{50}) after 5 min at 23 C was 1.75 nM (Table 22). This was three-fold higher than the IC_{50} for eel AChE, which was estimated to be 0.58 nM.

Data on the amount of HI-6 reactivation of ovine AChE at 23 and 37 C are presented in Figure 13 as a semi-logarithmic plot. The average log percent reactivation value for each time point was used to construct the plot. The average slope and intercept values from the regression analyses performed on the individual sheep log percent reactivation versus incubation time data are shown in Table 23. Statistical analysis of these data indicated significant differences ($P \leq 0.05$) in the slope and intercept values of the 23 C and 37 C data. The y-intercept (the estimated percent of AChE that can be reactivated by HI-6 at time zero) and slope values were significantly less ($P \leq 0.05$) at 37 C than at 23 C. Taking the inverse log of the y-intercept shows that at time zero there is 86.1 and 70.5 percent reactivation of AChE by HI-6 at 23 and 37 C, respectively. The more negative slope value obtained at 37 C is indicative of a faster rate of aging than was observed at 23 C. These data are not surprising in that the aging rate of cycloheptylmethylphosphonylated bovine RBC AChE has been shown to increase as the reaction temperature increases.[34]

Table 24 presents estimates of time required to obtain various levels of aging of GD-inhibited ovine AChE at incubation temperatures of 23 C and 37 C. Fifty percent of AChE activity appears to have aged after 3.2 min or 8.4 min of incubation at 37 and 23 C, respectively. This is a 2.5-fold difference in aging at the two temperatures. Twenty percent of the GD-inhibited AChE activity could be reactivated by HI-6 at 11.6 min at 37 C and 22.6 min at 23 degrees C.

FIGURE 12. INHIBITION OF OVINE RBC AND EEL ACHE BY QD

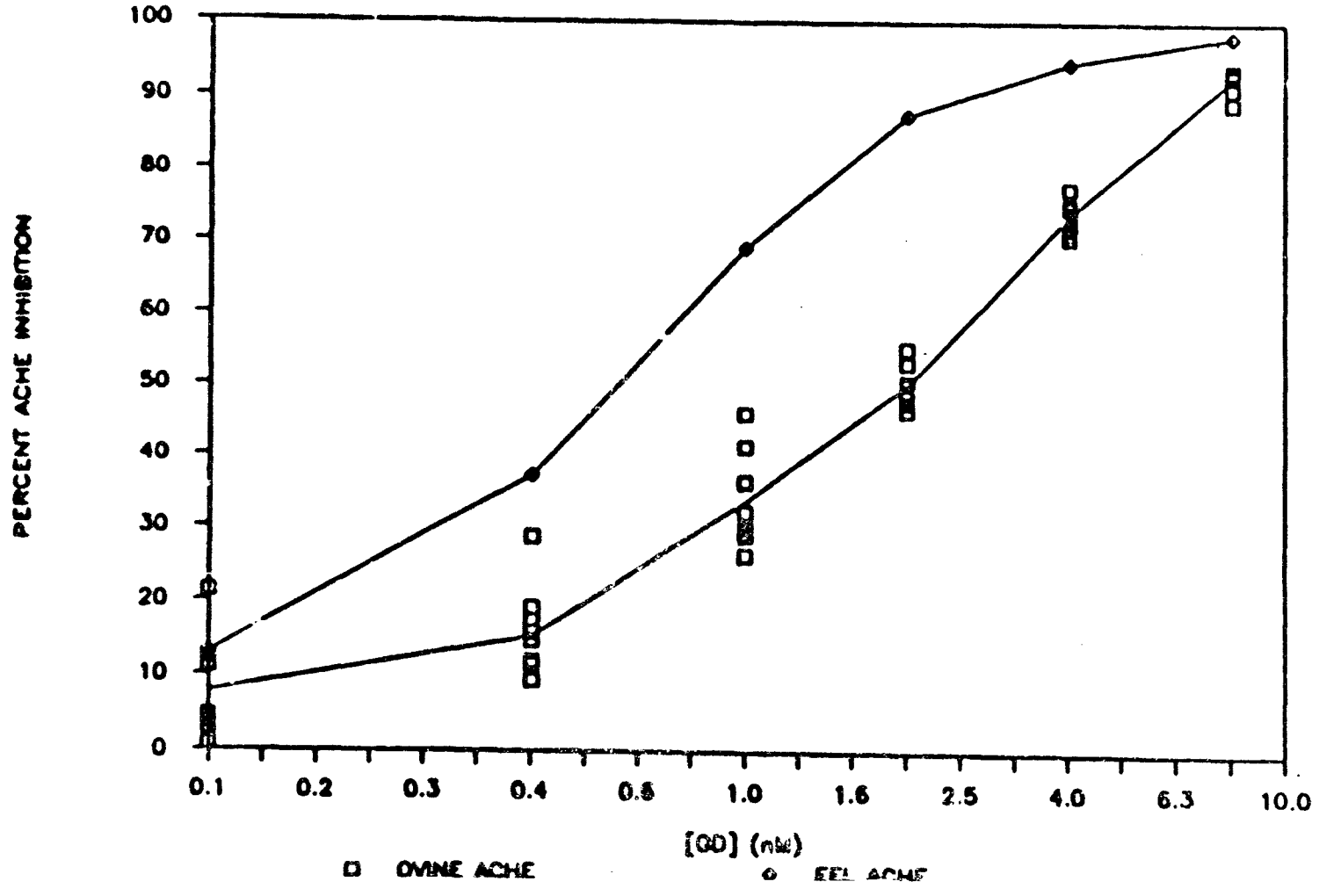


TABLE 22. ESTIMATION OF THE GD CONCENTRATION REQUIRED TO INHIBIT OVINE AChE BY FIFTY PERCENT (IC_{50}) AT 23 C[a]

SHEEP ID	IC_{50} VALUE [GD] mM
43	1.90
34	1.92
45	1.87
39	1.93
23	1.88
31	1.68
28	1.72
06	1.69
40	1.58
51	1.66
MEAN	1.75
STD	0.23
SEM	0.07

[a] The line equation (Y=MX+B) is used to solve for log [GD] given the level of percent inhibition. The individual slope (M) values and y-intercept (B) values were used in these calculations. Eel AChE exhibited an IC_{50} value of 0.58 mM GD.

FIGURE 13 REACTIVATION OF GD-INHIBITED AChE BY HI-6 AT 23 C AND 37 C

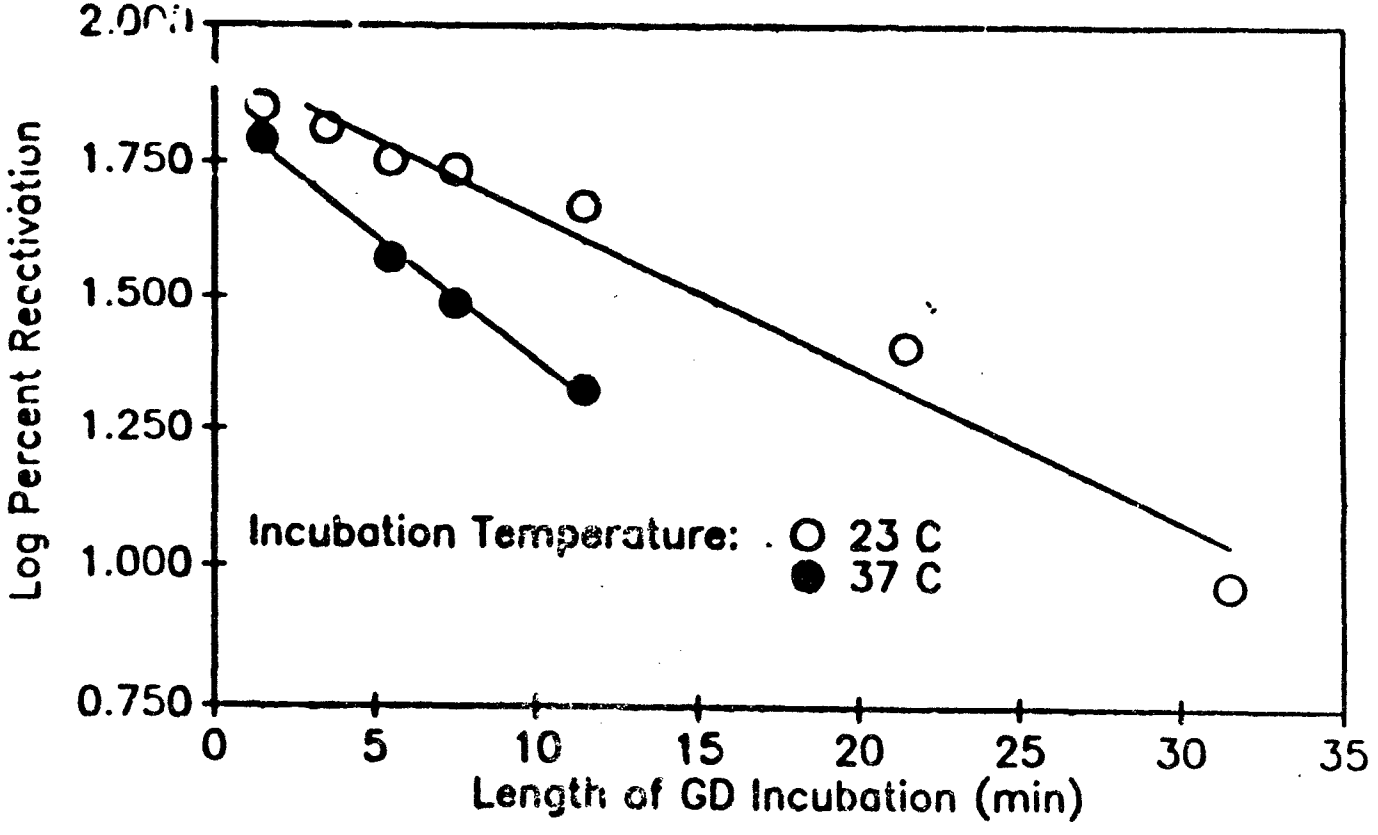


TABLE 23. SUMMARY OF THE SLOPE AND Y-INTERCEPT VALUES FROM REGRESSION ANALYSIS OF THE AGING DATA[a]

	SLOPE		Y-INTERCEPT	
	23 C	37 C	23 C	37 C
MEAN	-0.028	-0.047[b]	1.935	1.848[b]
STD	0.004	0.006	0.033	0.031
SEM	0.001	0.002	0.009	0.010

[a] The slope and y-intercept values are averages derived from individual regression analyses of aging data of blood samples from ten different sheep.

[b] Differs from 23 C value at $p \leq 0.05$.

TABLE 24. ESTIMATION OF GD-AChE INCUBATION TIME FOR VARIOUS AChE REACTIVATION VALUES[a]

PERCENT REACTIVATION	GD-AChE INCUBATION LENGTH (MINUTES)[b]	
	23 C	37 C
10.0	33.4	18.0
20.0	22.6	11.6
30.0	16.4	7.9
40.0	11.9	5.2
50.0	8.4	3.2
60.0	5.6	1.5
70.0	3.2	0.1
80.0	1.1	[c]

[a] Reactivator = 800 μM HI-6; GD = 11.8 nM

[b] Line Equation: $Y = M^*X + B$.

23 C: $X = (Y - 1.935)/(-0.028)$
37 C: $X = (Y - 1.848)/(-0.047)$

[c] Not attainable at 37 C.

4.0 CONCLUSIONS

The estimation of the GD 48-hr LD_{50} in the population of sheep used in this study did not differ statistically from the 6.6 µg/kg predicted in a previous task, and this value was accepted as the LD_{50} in this study. Statistical analysis of treatment efficacy data demonstrated that the 48-hr GD LD_{50} of sheep treated with atropine alone (7.1 µg/kg) was significantly less than the 48-hr GD LD_{50}s estimated for sheep treated with atropine/2-PAM (12.7 µg/kg) or atropine/HI-6. There were no statistical differences in LD_{50}s of sheep treated with atropine/HI-6 when using wet/dry autoinjectors (15.0 µg/kg), syringes (14.0 µg/kg), or Atropens and syringes (16.7 µg/kg). While the 48-hr GD LD_{50} estimated for sheep treated with atropine/2-PAM was not significantly less (at the 0.05 level) than the LD_{50}s of sheep treated with atropine/HI-6 using syringes or wet/dry autoinjectors, it was marginally different than the LD_{50} for sheep treated with atropine/HI-6 using Atropens and syringes. Since atropine/HI-6 treatments using various delivery techniques were not statistically different, the data was combined for the three HI-6 delivery systems and a 48-hr GD LD_{50} of 14.7 µg/kg was estimated for HI-6 treated sheep. Using this value, the PR for atropine/HI-6 treatment was 2.23 with a 95 percent confidence interval of 1.97 to 2.53.

Analysis of mortality data at 10 hr following injection of GD led to similar results in that the GD LD_{50} estimated for sheep treated only with atropine was less than for atropine/2-PAM or atropine/HI-6 treated sheep. There were no significant differences in LD_{50}s of sheep treated with atropine/HI-6 due to the use of different injection techniques, and there were no significant differences in LD_{50}s of atropine/2-PAM treated sheep and atropine/HI-6 treated sheep. Predicted times to death at 2 X 48-hr GD LD_{50} of untreated sheep (13.2 µg/kg) were significantly less for sheep treated only with atropine than for atropine/2-PAM and atropine/HI-6 treated sheep. Times to death for atropine/2-PAM and atropine/HI-6 treated sheep were not significantly different, although predicted times were numerically larger for atropine/HI-6 treated sheep.

In the modeling of signs of GD intoxication, durations of tremors and convulsions were positively related to GD dose, with durations increasing

with higher GD doses. This relationship was statistically significant when data from all sheep was modeled and was also significant for duration of convulsions when data only from 2-hr survivors was modeled. Analyses of durations of tremors and convulsions from all sheep revealed significant treatment group effects. Mean durations were shortest for HI-6/Atropen and longest for the untreated sheep. Though not statistically significant, this same relationship was observed among the group means when data were restricted to 2-hr survivors only. Duration of sternal recumbency among 48-hr survivors did not show any significant relationship with either GD dose or treatment group although inferences were limited due to the reduced sample size.

No unusual pathologic findings were noted. Observations of sheep for clinical signs of atropinization during pharmacokinetic studies yielded no information of significance.

Based on analyses of HI-6 and atropine pharmacokinetic parameters, there were no statistically significant differences between the two injection techniques. Numerically, mean values of the empirical and model-based estimates of $AUC_{0-\infty}$ and C_{max} were somewhat greater for syringe injection than for the wet/dry autoinjector for both HI-6 and atropine.

The aging rate of sheep red blood cell AChE is highly dependent upon the GD-AChE incubation temperature. Under the conditions used, fifty percent of sheep RBC AChE appears to age by 3.2 min at 37 C and by 8.4 min at 23 C. Twenty percent of GD-inhibited sheep RBC AChE could be reactivated by HI-6 at 11.6 min when incubated at 37 C and at 22.6 min when incubated at 23 C.

5.0 RECORD ARCHIVES

Of the 104 sheep used in Task 89-06, 55 arrived at the MREF on 3/6/90 and the remainder arrived on 4/10/90. LD_{50} and treatment efficacy studies were conducted between 3/14/90 and 6/13/90. Pharmacokinetic studies were conducted between 6/25/90 and 8/15/90. Records pertaining to the conduct of these studies are contained in Battelle laboratory record books which are specific for this task. These record books are clearly labeled as to contents of each volume. These records and the final report will be maintained at the MREF until acceptance of the final report by the U.S. Army. At that time,

records will be forwarded to the U.S. Army or archived at Battelle. Unused autoinjectors have been returned to their manufacturer or to USAMRICD.

6.0 ACKNOWLEDGMENTS

The names, titles and degrees of the principal contributors to this study are listed below:

Name	Title	Degree
Dr. Garrett S. Dill	Principal Investigator	D.V.M.
Dr. Carl T. Olson	Study Director	B.V.M., Ph.D.
Dr. Ronald G. Manton	Study Statistician	Ph.D.
Ms. Robyn C. Kiser	Study Supervisor	B.S.
Dr. James A. Blank	*In Vitro* Pharmacologist	Ph.D.
Mr. Thomas H. Snider	Pharmacokinetics Modeler	B.S.
Ms. M. Claire Matthews	Statistician	M.A.
Mr. Timothy L. Hayes	Study Chemist	B.A.
Dr. Larry S. Miller	Immunologist	Ph.D.
Dr. Ronald L. Persing, Jr.	Study Pathologist	D.V.M.
Dr. Peter L. Jepsen	Study Veterinarian	B.V.M.

There are a number of people who made performance of this task possible. Their invaluable assistance is gratefully acknowledged by the authors. Among the many are: James Arp and Sheri Moore for chemical analyses; Rebekah Starner and Mary Lou Brimer for *in vitro* aging analyses; Victor Moore for RIA analyses; Drs. John D. Toft, II and Allen W. Singer and Mr. Anthony Stuart for pathology support; Kandy Audet and Michael Hingson, and Linda Adams, Karen Brown, Pamela Cooley, Rebecca Geer, William Hart, Pamela Kinney, Jonathon Kohne, Joan Ostovich, Cynthia Polley, and Jack Waugh for preparation of the sheep and performance of technical tasks; and Charlotte Hirst and Tami Kay for preparation of the report.

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APPENDIX A

Protocols

Determination of the Efficacy of HI-6 and Atropine
as Delivered by a Wet/Dry Autoinjector or by Syringe
in the Treatment of Soman Intoxication in Sheep

Study performed by Battelle Memorial Institute
505 King Avenue, Columbus, Ohio 43201-2693

1. Program Manager: Garrett S. Dill, D.V.M.
2. Study Director: Carl T. Olson, D.V.M., Ph.D.
3. Veterinarian: Peter L. Jepsen, D.V.M.
4. Statistician: Ronald G. Manton, Ph.D.
5. Pathologist: Allen W. Singer, D.V.M.
6. Sponsor: U.S. Army Medical Research and Development Command (USAMRDC)
7. Sponsor Monitor: MAJ James R. Stewart, Ph.D., U.S. Army Medical Research Institute of Chemical Defense (USAMRICD)
8. Objective: The oxime HI-6 given in conjunction with atropine appears to be the most effective treatment of pinacolyl methylphosphonofluoridate (Soman; GD) intoxication available. The efficacy of HI-6 against GD has been repeatedly demonstrated in a variety of animals, including rodents, rabbits, dogs, and nonhuman primates. A major obstacle to the development of HI-6 for military use is its marked instability in solution. Current U.S. Army autoinjector systems package all components in solution. An effort is underway to develop a delivery device, a wet/dry autoinjector, which will allow packaging of HI-6 in its stable, crystalline form. Two companies have nearly completed development of systems which allow the packaging of HI-6 in dry form until just prior to use. In these systems, HI-6 is rapidly solvated by a solution of atropine so that within a matter of seconds the mixture of atropine and HI-6 can be injected. Since adequate shelf life (> 3 yr) can be expected for HI-6 while in a dry form, the use of a wet/dry autoinjector appears absolutely critical to the successful fielding of this oxime as a nerve agent antidote.

   No in vivo studies have been reported that document the wet/dry autoinjector concept as valid for an emergency antidote approach for nerve agent intoxication. Substantial differences exist between the experimental administration of previously prepared solutions of atropine plus HI-6 given by syringe and the proposed administration of the drugs using a wet/dry autoinjector. The important potential problems with a wet/dry autoinjector delivery include:

- Any measurable delay in the administration of nerve agent antidote because of the required solubilization time for HI-6 may result in therapeutic failure. Time to treatment becomes critical once signs of nerve agent intoxication are manifested.

- An atypical dispersion pattern of the admixed solution in tissue may markedly alter the absorption of the antidote components. The design of the autoinjectors is such that the tissue dispersion pattern of the injected material cannot be simulated in the laboratory with the use of syringes. This atypical pattern has been shown to alter the pharmacokinetic behavior of atropine when compared to conventional injection methods.

- A relatively small deliverable dose of HI-6 due to a small, fixed volume capacity of the autoinjector may be inadequate. Although the data base for HI-6 suggests efficacy against GD intoxication, virtually all studies have been done in small animals (< 10 kg) at relatively large doses (20-200 mg/kg). As packaged in available autoinjectors, the dose of HI-6 to a 70 kg man ranges from 5.7 to 7.1 mg/kg per injection. It is not certain that HI-6 can provide improved survivability or reduced morbidity relative to the currently fielded nerve agent antidote regimen when its use is constrained by formulation and/or doctrinal considerations.

The objectives of this task are to:

- Determine efficacy of treatment of GD intoxication in sheep with atropine and HI-6 delivered intramuscularly (IM) by wet/dry autoinjector compared to delivery by conventional hypodermic syringe.

- Compare efficacy of atropine/HI-6 delivered by wet/dry autoinjector to the efficacy of atropine plus pralidoxime chloride (2-PAM) delivered by the Mark I (MKI) autoinjector in preventing the effects of GD intoxication in sheep.

9. Experimental Design:

A. Test System

(1) Animals - Sheep are used for this study because of their known response to organophosphate (OP) chemical surety materiel (CSM), and because of similarities with man in body weight. Experiments are conducted in a stage-wise fashion to limit the number of animals used to the minimum necessary to achieve statistically valid results. Sheep are held for up to 48 hr following exposure, at which time survivors are euthanatized by the injection of high concentrations of pentobarbital sodium. Discomfort and injury of

animals are limited to that which is unavoidable in the conduct of scientifically valuable research. If, in the judgement of the Study Director or the Study Veterinarian, a sheep appears to be in a moribund state and in pain, that animal will be euthanatized. However, since animals moribund during the first 24 hr following OP exposure have been observed to fully recover, no animals will be euthanatized during this period. Anesthetics, analgesics, or tranquilizers for the relief of pain or anxiety cannot be used in these studies because they would interfere with the biological effects caused by the challenge agent or test compounds. External stimuli and manipulation are minimized to decrease any associated anxiety.

Protocols of all experiments using animals are reviewed and approved by Battelle's Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study. The Program Manager accepts responsibility for the proper care and use of animals in the conduct of research described in the protocols.

Sheep are serologically Q-Fever negative, mature wethers obtained from Thomas D. Morris, Inc. (Reistertown, MD) or another similar, approved source of research animals. Sheep are shorn, as necessary, to improve their comfort in an indoor environment.

(2) Weight - Initial weight of sheep will be 60-80 kg.

(3) Quarantine - Sheep are examined by a veterinarian upon arrival. Blood samples are drawn for complete blood counts and fecal samples are obtained for parasite infestation evaluation. Sheep are held in isolation and observed for signs of clinical illness for at least 7 days prior to use in a study.

(4) Selection - Animals selected after quarantine are in good physical condition. Sheep are weighed, and, based on weight, randomly assigned utilizing a program (AMRAND) written at Battelle for use on personal computers, that assures homogeneity across groups. A maximum of 95 sheep will be used in this efficacy study.

(5) Animal Identification - All animals are tagged in the ear to retain positive identification during handling and observation.

(6) Housing - Sheep are group housed in an outdoor fenced area with available shelter. Prior to experimentation, they are brought into the laboratory and maintained as small groups in animal holding rooms. At the time of experimentation, they will be placed in slings to which they have been acclimated.

(7) Lighting - Sheep are group housed in an outdoor fenced area prior to experimentation. When they are moved into experimental areas, fluorescent lighting with a light/dark cycle of 12 hr each per day is used.

(8) Temperature - Maintained at 70 ± 10 F in indoor areas.

(9) Humidity - Maintained at 50 ± 10 percent in indoor areas.

(10) Diet - Sheep are fed Purina Rumilab Chow and a limited amount of hay. If necessary to maintain or increase body weight, a higher energy feed such as a mixture of cracked corn, alfalfa pellets and molasses may be locally procured and fed. No contaminants that would interfere with the results of the study are known to be present in the feed.

(11) Water Supply - Water is supplied from the Battelle West Jefferson water system and given ad libitum during quarantine and holding. No contaminants that would affect the results of the study are known to be present in the water. Water is analyzed for impurities on an annual basis.

(12) Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-21) since August 14, 1967, and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. In addition, animals for use in research are obtained only from laboratory animal suppliers duly licensed by the USDA. Battelle's most recent statement of assurance regarding the Department of Health and Human Services (DHHS) policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institutes of Health on July 29, 1986. Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (DHHS Publication No. (NIH) 85-23) and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, pursuant to the Laboratory Animal Welfare Act of August 24, 1966 as amended (P.L. 89-544 and P.L. 91-579).

(13) On January 31, 1978, Battelle's Columbus operations received full accreditation of its animal care programs and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

B. Test Material

(1) Treatment Compounds - Treatment compounds, atropine, and 2-PAM, contained in MKI antidote injection systems and atropine and HI-6 contained in wet/dry autoinjectors as well as additional HI-6 are provided by USAMRICD. Commercially available injectable atropine sulfate solutions are purchased for use as supplemental atropine in efficacy studies. Sufficient numbers of each injection system from the same lot are provided so that sheep can be injected to perform efficacy experiments against GD challenge.

(2) Chemical Agents - GD is supplied by USAMRICD. Purity, appropriate identification (batch number, lot number, state), and stability data are supplied by USAMRICD. Purity and stability of agents stored at Battelle are periodically confirmed by Battelle personnel using analytical chemistry techniques and standards provided by USAMRDC.

(3) Surety, security, and safety procedures for the use of OP chemical agents are thoroughly outlined in facility plans, in personnel requirements for qualification to work with CSM, and in standard operating procedures (SOPs) for storage and use of CSM.

C. Test Groups - Experiments are carried out in two distinct phases. The first phase is to estimate a 24 hr GD LD_{50} in this population of sheep. Prior experimentation in MREF Task 88-38 established the 24 hr GD LD_{50} in sheep at approximately 6.5 µg/kg body weight. Sheep are given IM injections of GD at doses established by statisticians using only a few sheep a day in an up-and-down design. Using a common slope model (i.e., using the slope determined for the dose-response lethality curve for GD determined in MREF Task 88-38), an LD_{50} is estimated by a probit model. A statistical hypothesis test is then conducted to determine if the two LD_{50}s are similar. No more than 10 sheep are used in this phase. If after five or more sheep have been dosed, the estimated LD_{50} is within the 95 percent confidence limits of the LD_{50} obtained in MREF Task 88-38, the LD_{50} estimated in MREF Task 88-38 is accepted as the LD_{50} for sheep in this study. The mean onset time (MOT) of cholinergic signs of OP intoxication in sheep given GD LD_{50} doses or greater is estimated.

Phase two is accomplished to compare the GD intoxication treatment efficacies of atropine/HI-6 given by 3 wet/dry autoinjectors, equivalent doses of atropine/HI-6 given by syringe, and atropine/2-PAM given by 3 MKI autoinjectors. Treatments are given at the MOT determined in phase one or at a time selected by the Study Director and the USAMRICD technical contact for this study. The dose of atropine is augmented via syringe to provide 0.5 mg of atropine

sulfate equivalents per kilogram of body weight. Experiments are conducted in a stagewise fasnion, using only a few sheep in each treatment group per day, to determine the LD_{50} for each group. The LD_{50}s and slopes of the dose response curves are compared after each stage to determine any significant differences in efficacies of therapy. A maximum of 25 sheep in the atropine/HI-6 via syringe and atropine/2-PAM (MKI) treatment groups are used. A maximum of 35 sheep are used in the atropine/HI-6 via wet/dry autoinjector group. Since this atropine/HI-6 group LD_{50} will be compared to both the atropine/HI-6 by syringe LD_{50} and the atropine/2-PAM MKI autoinjector LD_{50}, the power of statistical comparisons is increased by dosing additional sheep in this group. If significant differences (α of 0.05) in treatment efficacies is determined with fewer sheep, experimentation will cease at that time. If after a minimum of 10 sheep in each of the three groups have been challenged and treated there is not a statistical difference in LD_{50} values of atropine/2-PAM and atropine/HI-6 treated sheep, the USAMRICD Task Area Manager and Contracting Officer's Representative are consulted and a course of further action planned.

D. Study Preparations - Animals are held in a pen and acclimated to a sling at the MREF prior to use. Each sheep is weighed within 24 hr of intended use. Anesthesia is not used since it would affect the occurrence of clinical signs of OP intoxication.

E. Injection of Challenge and Treatment Materials - Animals are restrained in slings at the time of dosing. Dosing of exempt concentrations of GD will occur at the face of an approved hood, with no more than 2 mL injected at any site in the caudal muscles of the right thigh and with no more than two injection sites. Treatments are given in the anterior lateral muscles of the left thigh with a 1 inch or more separation of injection sites.

F. Observations - Sheep are observed for signs of GD intoxication, including muscle fasciculations, tremors, convulsions, excessive salivation/bronchial secretions, and prostration, as feasible, for 48 hr following GD challenge. Animals are closely observed for signs of intoxication for the first 2 hr following agent challenge, and then at decreasing frequency for the remainder of the 48-hr observation period. Any signs observed and the time of onset are recorded as feasible.

G. Disposal of Experimental Animals - Animals dying on study are necropsied and gross lesions recorded. After necropsy, remains are incinerated. Sheep still alive at the end of the 48-hr observation period are euthanatized and cremated. No tissue sample collection is performed.

10. Statistical Approach: Estimates of the GD dose-24 hr lethal response relations for GD in sheep given atropine/HI-6 via wet/dry autoinjector are compared with those of sheep given atropine/HI-6 by syringe c atropine/2-PAM by MKI autoinjector to determine if significant differences ($P < 0.05$) exist between efficacies of the treatments. Sheep are used in a stage-wise design to minimize the number of animals used to determine if significant differences exist. Two parameter probit dose-response models are fitted separately to each treatment group by means of maximum likelihood estimation to predict the relation between probability of lethality within 24 hr and GD dose. Comparison of the three treatments is accomplished by testing for significant differences in estimated LD_{50}s and slope values obtained for the treatments. Based on these dose-response fits, estimates of the LD_{50} dose level and slope and associated 95 percent confidence intervals are made for each treatment. If statistically significant differences ($P < 0.05$) between injection systems are detected with fewer animals than the established maximum, experimentation ceases at that time.

The incidence or recovery from sublethal responses, such as convulsions or prostration, are statistically analyzed, as feasible, to determine differences in incidence or severity of effects in sheep treated differently.

11. Records to be Maintained:

A. CSM accountability log and inventory

B. Preparation of reagents and dosage administration

C. Animal response data

D. Experimental parameters and test conditions

E. Confirmation of disposal

12. Reports:

A Draft Final Report will be prepared and submitted for review to the USAMRDC COR within 60 working days after completion of the task. It will include the following:

A. Signature page for key study individuals and their responsibilities

B. Experimental design

C. Animal supplier

D. Test animal selection criteria

E. Test material description and preparation

F. Clinical observations

G. Tabulation of response data

H. Statistical methodology

I. Discussion

A Final Report that addresses the review comments of USAMRDC will be prepared and submitted within 30 working days of receipt of comments.

13. Approval Signatures:

Carl T. Olson
Carl T. Olson, D.V.M., Ph.D.
Study Director
10-4-89
Date

Garrett S. Dill, D.V.M.
Program Manager
10/4/89
Date

Peter L. Jeyson
Peter L. Jeyson, D.V.M.
Study Veterinarian
10/13/89
Date

Ronald Menton
Ronald G. Menton, Ph.D.
Statistician
10/17/89
Date

Allen W. Singer, D.V.M.
Pathologist

10-18-89
Date

MAJ James R. Stewart, Ph.D.
USAMRICD COR

12 Oct 89
Date

Ramona A. Mayer, B.S.
Manager, Quality Assurance

Dec. 4, 1989
Date

Anna O. Barker, Ph.D.
Vice President
Health and Environment Group

11/7/89
Date

MREF Protocol 53
Medical Research and
Evaluation Facility
March 14, 1990

Determination of the Efficacy of HI-6 and Atropine as Delivered by a Wet/Dry Autoinjector or by Syringe in the Treatment of Soman Intoxication in Sheep

Protocol Amendment No. 1

Change: Page 4, Section 9.A.(8).

Temperature will be maintained at 65 ± 15 F in indoor areas.

Reason: Change is made to be consistent with MREF SOP C-08-4, April 1989.

Impact on Study: None.

Change: Page 4, Section 9.A.(9).

Relative humidity will be maintained at 50 ± 20 percent in indoor areas.

Reason: Change is made to be consistent with MREF SOP C-08-4, April 1989.

Impact on Study: None.

Carl T. Olson

Carl T. Olson, D.V.M., Ph.D.
Study Director

3-14-90
Date

Don W. Korte, Jr.

LTC Don W. Korte, JR
USAMRICD COR

15 March 90
Date

Determination of the Efficacy of HI-6 and Atropine
as Delivered by a Wet/Dry Autoinjector or by Syringe
in the Treatment of Soman Intoxication in Sheep

Protocol Amendment No. 2

Change: Page 5, Section 9.C.

"Treatments are given at the MOT determined in phase one or at a time selected by the Study Director and the USAMRICD technical contact for this study." is replaced with "Treatment injections are given as simultaneously as possible at 1 min after GD injection."

Reason: Time and method of injection were requested by MAJ James R. Stewart, USAMRICD.

Impact on Study: None.

Carl T. Olson, D.V.M., Ph.D.
Study Director

3-29-90
Date

LTC Don W. Korte, Jr.
USAMRICD COR

30 Mar 90
Date

Determination of the Efficacy of HI-6 and Atropine
as Delivered by a Wet/Dry Autoinjector or by Syringe
in the Treatment of Soman Intoxication in Sheep

Addendum 1

After sheep have been dosed with GD, and 14 treated with atropine and HI-6 using wet/dry autoinjectors, and 10 each treated with either Mark I autoinjectors or atropine and HI-6 by syringe, there is no statistical difference in the LD_{50}s of GD between treatments, although the estimated LD_{50}s are more than twice that of sheep given no therapy. As described in the protocol, the USAMRICD Contracting Officer's Representative (COR) and Task Area Manager (TAM) were consulted when this occurred. As a result, additional treatment groups will be used. This may include similar groups given the same treatments by separate injections of atropine and HI-6 (i.e., not admixed) or different treatments such as increased or decreased levels of atropine to determine if efficacy of the HI-6 changes, or treatments of atropine alone without an oxime to determine any changes in LD_{50}s. This should help delineate effectiveness of HI-6 in treatment of GD toxicity in sheep.

Carl T. Olson

Carl T. Olson, D.V.M., Ph.D.
Study Director

4-19-90
Date

Don W Korte, Jr.

LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

19 APR 90
Date

Determination of the Efficacy of HI-6 and Atropine
as Delivered by a Wet/Dry Autoinjector or by Syringe
in the Treatment of Soman Intoxication in Sheep

Deviation: This protocol specifies sheep will be held in rooms with a temperature range of 50-80 F and a relative humidity of 30-70 percent. Conditions in animal rooms are recorded twice daily using a hand-held combination thermometer/hygrometer to obtain temperature and relative humidity readings. The relative humidity recorded in rooms in which sheep were held during the efficacy phase of the experiment ranged from 29 to 77 percent. Excursions outside relative humidity ranges specified in the protocol were reported to a maintenance engineer and adjustments of humidistats made.

Impact on Study: Temperature and relative humidity ranges recommended for sheep are not specified by the National Institutes of Health in their Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, Revised 1985). The short-lived excursions outside relative humidity specifications stated in the protocol should have no impact on the validity of the study.

Carl T. Olson

Carl T. Olson, D.V.M., Ph.D.
Study Director

10-18-90
Date

Don W. Korte

LTC Don W. Korte, Jr., M.S.
USAMRICD COR

18 OCT 90
Date

Determination of the Efficacy of HI-6 and Atropine
as Delivered by a Wet/Dry Autoinjector or by Syringe
in the Treatment of Soman Intoxication in Sheep

Protocol Amendment No. 3

Change: Page 5, Section 9.C.

"The first phase is to estimate a 24 hr GD LD_{50} in this population of sheep." is changed to read "The first phase is to estimate a 48 hr GD LD_{50} in this population of sheep."

Page 7, Section 10.

"Estimates of the GD dose-24 hr lethal response ....." is changed to read "Estimates of the GD dose-48 hr lethal response ....."

Reason: Sheep are being held and observed for 48 hr, and to be consistent with statistical analyses performed in previous tasks, it is advisable to base analyses on 48 hr rather than 24 hr mortality.

Impact on Study: This should create no effect on the performance of the study other than the data analyzed. In previous experiments in sheep, there has been virtually no difference in 24 hr and 48 hr GD LD_{50} values.

Carl T. Olson

Carl T. Olson, D.V.M., Ph.D.
Study Director

11-2-90
Date

Don W. Korte, Jr.

LTC Don W. Korte, Jr.
USAMRICD CCR

2 Nov 90
Date

Comparison of the Pharmacokinetics of HI-6 and Atropine as Delivered by a Wet/Dry Autoinjector or by Syringe

Study performed by Battelle Memorial Institute
505 King Avenue, Columbus, Ohio 43201-2693

1. Program Manager: Garrett S. Dill, D.V.M.
2. Study Director: Carl T. Olson, D.V.M., Ph.D.
3. Veterinarian: Peter L. Jepsen, D.V.M.
4. Statistician: Ronald G. Menton, Ph.D.
5. Sponsor: U.S. Army Medical Research and Development Command (USAMRDC)
6. Sponsor Monitor: MAJ James R. Stewart, Ph.D., U.S. Army Medical Research Institute of Chemical Defense (USAMRICD)
7. Objective: The oxime HI-6 given in conjunction with atropine appears to be the most effective treatment of pinacolyl methylphosphonofluoridate (Soman; GD) intoxication available. The efficacy of HI-6 against GD has been repeatedly demonstrated in a variety of animals, including rodents, rabbits, dogs, and nonhuman primates. A major obstacle to the development of HI-6 for military use is its marked instability in solution. Current U.S. Army autoinjector systems package all components in solution. An effort is underway to develop a delivery device, a wet/dry autoinjector, which will allow packaging of HI-6 in its stable, crystalline form. Two companies have nearly completed development of systems which allow the packaging of HI-6 in dry form until just prior to use. In these systems, HI-6 is rapidly solvated by a solution of atropine so that within a matter of seconds the mixture of atropine and HI-6 can be injected. Since adequate shelf life (> 3 yr) can be expected for HI-6 while in a dry form, the use of a wet/dry autoinjector appears absolutely critical to the successful fielding of this oxime as a nerve agent antidote.

No in vivo studies have been reported that document the wet/dry autoinjector concept as valid for an emergency antidote approach for nerve agent intoxication. Substantial differences exist between the experimental administration of previously prepared solutions of atropine plus HI-6 given by syringe and the proposed administration of the drugs using a wet/dry autoinjector. The important potential problems with a wet/dry autoinjector delivery include:

- Any measurable delay in the administration of nerve agent antidote because of the required solubilization time for HI-6 may result in therapeutic failure. Time to treatment becomes critical once signs of nerve agent intoxication are manifested.

- An atypical dispersion pattern of the admixed solution in tissue may markedly alter the absorption of the antidote components. The design of the autoinjectors is such that the tissue dispersion pattern of the injected material cannot be simulated in the laboratory with the use of syringes. This atypical pattern has been shown to alter the pharmacokinetic behavior of atropine when compared to conventional injection methods.

The major objective of this task is to compare the pharmacokinetics of HI-6 and atropine when delivered by a wet/dry autoinjector to those when atropine and HI-6 are delivered by a conventional hypodermic syringe. A second objective is to determine the aging rate of GD in sheep erythrocytes (RBCs).

8. Experimental Design:

A. Test System

(1) Animals - Sheep are used for this study because of previous studies on the pharmacokinetics of atropine and HI-6 in this species and because of similarities with man in body weight.

Protocols of all experiments using animals are reviewed and approved by Battelle's Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study. The Program Manager accepts responsibility for the proper care and use of animals in the conduct of research described in the protocols.

Sheep are serologically Q-Fever negative, mature wethers obtained from Thomas D. Morris, Inc. (Reistertown, MD) or another similar, approved source of research animals. Sheep are shorn, as necessary, to improve their comfort in an indoor environment.

(2) Weight - Initial weight of sheep will be 60-80 kg.

(3) Quarantine - Sheep are examined by a veterinarian upon arrival. Blood samples are drawn for complete blood counts and fecal samples are obtained for parasite infestation evaluation. Sheep are held in isolation and observed for signs of clinical illness for at least 7 days prior to use in a study.

(4) Selection - Animals selected after quarantine are in good physical condition. Sheep are weighed, and, based on weight, randomly assigned utilizing a program (AMRAND) written at Battelle for use on personal computers that assures homogeneity across groups. Eight sheep will be used in this pharmacokinetic study.

(5) Animal Identification - All animals are tagged in the ear to retain positive identification during handling and observation.

(6) Housing - Sheep are group housed in an outdoor fenced area with available shelter. Prior to experimentation, they are brought into the laboratory and maintained as a small group in an animal holding room. At the time of experimentation, they will be placed in slings to which they have been acclimated.

(7) Lighting - Sheep are group housed in an outdoor fenced area prior to experimentation. When they are moved into experimental areas, fluorescent lighting with a light/dark cycle of 12 hr each per day is used.

(8) Temperature - Maintained at 70 ± 10 degrees F in indoor areas.

(9) Humidity - Maintained at 50 ± 10 percent in indoor areas.

(10) Diet - Sheep are fed Purina Rumilab Chow and a limited amount of hay. If necessary to maintain or increase body weight, a higher energy feed such as a mixture of cracked corn, alfalfa pellets and molasses may be locally procured and fed. No contaminants that would interfere with the results of the study are known to be present in the feed.

(11) Water Supply - Water is supplied from the Battelle West Jefferson water system and given ad libitum during quarantine and holding. No contaminants that would affect the results of the study are known to be present in the water. Water is analyzed for impurities on an annual basis.

(12) Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-21) since August 14, 1967, and are periodically inspected in accordance with the provisions of the Federal Animal

Welfare Act. In addition, animals for use in research are obtained only from laboratory animal suppliers duly licensed by the USDA. Battelle's most recent statement of assurance regarding the Department of Health and Human Services (DHHS) policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institutes of Health on July 29, 1986. Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (DHHS Publication No. (NIH) 85-23) and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, pursuant to the Laboratory Animal Welfare Act of August 24, 1966 as amended (P.L. 89-544, P.L 91-579, and P.L. 99-198).

(13) On January 31, 1978, Battelle's Columbus operations received full accreditation of its animal care programs and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

B. Test Material

Treatment Compounds - Treatment compounds, atropine and HI-6, contained in wet/dry autoinjectors as well as additional HI-6 and atropine in the forms present in autoinjectors are provided by USAMRICD. Sufficient numbers of the wet/dry injection system from the same lot are provided so that sheep can be injected to perform pharmacokinetic experiments.

C. Test Groups

Sheep are given atropine/HI-6 IM with either three wet/dry autoinjectors or equivalent amounts with a conventional syringe. Dosages delivered by syringe will be in the same formulation as in autoinjectors and quantities of atropine and HI-6 based on average autoinjector dose as determined by USAMRICD. Concentrations will be specified in an amendment to this protocol and verified by HPLC analysis. After a minimum one week washout period, the same sheep are injected again using the injection technique not used originally. On each day of experimentation, an equal number of sheep will be given HI-6 and atropine by each of the two injection techniques. A total of eight sheep are given atropine/HI-6 with both injection systems. At times after injection of approximately 1, 2, 3, 4, 5, 6, 8, 12, 16, 20, 40, 60, 80, 120, 180, 240, 300, and 360 min, blood samples are taken from the jugular vein, either through an indwelling catheter or by using a syringe and needle.

Sheep will be observed for signs of atropinization, including pupillary size and response to light, ambulation when removed from slings, and changes from preinjection heart rate at approximately 20 min after injection.

Blood samples are analyzed for atropine and HI-6 concentrations. HI-6 concentrations are measured by Battelle using high performance liquid chromatography (HPLC) analyses and a standard HI-6 curve prepared from known concentrations. Atropine analyses are conducted at Battelle using radioimmunoassay techniques.

When atropine and HI-6 analyses are completed, blood concentrations as a function of time, maximum concentrations, times to maximum concentrations, area under the blood concentration-time curves, absorption and elimination rate constants, and volumes of distribution are estimated. Statistical analyses, as described in Section 9, are performed to determine if any significant differences exist between values as a function of the injection system.

D. Study Preparations

Animals are held in a pen and acclimated to a sling at the MREF prior to use. Each sheep is weighed within 24 hr of intended use.

E. Estimation of GD Aging Rate in Sheep RBCs

Estimation of the GD aging rate in sheep erythrocytes will be accomplished in vitro. Sheep RBCs will be incubated with a dilution of GD for various lengths of time and the ability of HI-6 to reactivate acetylcholinesterase (AChE) will be measured using an automated analysis as described in MREF SOP-88-46. RBCs from approximately 10 sheep will be analyzed to determine varability in aging rate.

9. Statistical Approach:

Pharmacokinetic parameters measured for atropine/HI-6 administered by the wet/dry autoinjector system are compared to those obtained for atropine/HI-6 administered by syringe to determine any significant ($P < 0.05$) differences. Responses will be analyzed using two-way crossover design analysis of variance techniques or t-tests.

10. Records to be Maintained:

A. Analyses of atropine and HI-6 in injection systems

B. Analyses of atropine and HI-6 in blood

C. Experimental parameters, test conditions, and observations for signs of atropinzation

D. Parameters and results of GD aging experiments

11. Reports:

A draft final report will be prepared and submitted for review to the USAMRDC COR within 60 working days after receipt of all analytical results. It will include the following:

A. Experimental design

B. Animal supplier

C. Pharmacokinetic and statistical methodolngy

D. Discussion of results.

A final report that addresses the review comments of USAMRDC will be prepared and submitted within 30 working days of receipt of comments.

12. Approval Signatures:

Carl T. Olson, D.V.M., Ph.D.
Study Director — Date: 20 Nov 89

Garrett S. Dill, D.V.M.
Program Manager — Date: 11/30/89

Peter L. Jepson, D.V.M.
Study Veterinarian — Date: 12/1/89

Ronald G. Menton, Ph.D.
Statistician — Date: 12/11/89

MAJ James R. Stewart, Ph.D.
USAMRDC COR

11-29-89
Date

Ramona A. Mayer, B.S.
Manager, Quality Assurance

12/12/89
Date

Anne O. Barker, Ph.D.
Vice President
Health and Environment Group

12/13/89
Date

Comparison of the Pharmacokinetics of HI-6 and Atropine as Delivered by a Wet/Dry Autoinjector or by Syringe

Protocol Amendment No. 1

Change: Page 4, Section 8.C.

Concentration of atropine free base will be approximately 0.59 mg/mL and concentration of HI-6 will be approximately 145.94 mg/mL in a total volume of approximately 3.15 mL.

Reason: U.S. Army Institute of Chemical Defense has analyzed Astra Wet/Dry autoinjector contents and reported a volume delivered of 3.15 mL (± 0.11) with 1.86 mg (± 0.07) atropine free base and 459.7 mg (± 9.86) HI-6.

Impact on Study: There is no impact of this amendment on the study. The protocol stated that concentrations of these compounds for use in the study would be specified in an amendment.

Carl T. Olson

Carl T. Olson, D.V.M., Ph.D.
Study Director

8 Feb 90
Date

Don W. Korte

LTC Don W. Korte, Jr., Ph.D., D.A.B.T.
USAMRICD COR

8 Feb 90
Date

MREF Protocol 54
Medical Research and
Evaluation Facility
March 14, 1990

Comparison of the Pharmacokinetics of HI-6 and Atropine as Delivered by a Wet/Dry Autoinjector or by Syringe

Protocol Amendment No. 2

Change: Page 4, Section 8.A.(8).

Temperature will be maintained at 65 ± 15 F in indoor areas.

Reason: Change is made to be consistent with MREF SOP C-08-4, April 1989.

Impact on Study: None.

Change: Page 4, Section 8.A.(9).

Relative humidity will be maintained at 50 ± 20 percent in indoor areas.

Reason: Change is made to be consistent with MREF SOP C-08-4, April 1989.

Impact on Study: None.

Carl T. Olson

Carl T. Olson, D.V.M., Ph.D.
Study Director

3-16-90
Date

Don W. Korte

LTC Don W. Korte, JR.
USAMRICD COR

16 March 90
Date

MREF Protocol 54
Medical Research and
Evaluation Facility
June 11, 1990

Comparison of the Pharmacokinetics of HI-6 and Atropine
as Delivered by a Wet/Dry Autoinjector or by Syringe

Protocol Amendment No. 3

Change: Page 5, Section 8.D.

"Each Sheep is weighed within 24 hr of intended use." is replaced with "Sheep will be randomized for treatment such that body weight will not bias the data."

Reason: Weighing sheep immediately prior to each study is unnecessary since each animal will be given both treatments in a cross-over design. Injections will be given in a random fashion as designed by a statistician in order to preclude effects of day of injection on pharmacokinetic parameters.

Impact on Study: None.

Carl T. Olson

Carl T. Olson, D.V.M., Ph.D.
Study Director

11 June 90
Date

Don W. Korte, Jr.

LTC Don W. Korte, JR
USAMRICD COR

11 June 90
Date

Comparison of the Pharmacokinetics of HI-6 and Atropine
as Delivered by a Wet/Dry Autoinjector or by Syringe

Deviation: This protocol specifies sheep will be held in rooms with a temperature range of 50-80 F and a relative humidity of 30-70 percent. Conditions in animal rooms are recorded twice daily using a hand-held combination thermometer/hygrometer to obtain temperature and relative humidity readings. The relative humidity recorded in rooms in which sheep were held during the pharmacokinetics phase of the experiment ranged as high as 81 percent. Excursions above the relative humidity range specified in the protocol were reported to a maintenance engineer and adjustments of humidistats made.

Impact on Study: Temperature and relative humidity ranges recommended for sheep are not specified by the National Institutes of Health in their Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, Revised 1985). The short-lived excursions outside relative humidity specifications stated in the protocol should have no impact on the validity of the study.

Carl T. Olson

Carl T. Olson, D.V.M., Ph.D.
Study Director

10-18-90
Date

Don W. Korte, Jr.

LTC Don W. Korte, Jr., M.S.
USAMRICD COR

18 OCT 90
Date

Comparison of the Pharmacokinetics of HI-6 and Atropine
as Delivered by a Wet/Dry Autoinjector or by Syringe

Deviation: Protocol Amendement No. 1 specified concentrations of atropine and HI-6 in solution to deliver desired doses to sheep in pharmacokinetic studies. Prior to the start of pharmacokinetic studies, an error in the information was detected. The atropine dose of 1.86 mg is an atropine sulfate equivalent dose rather than an atropine free base dose. The 460 mg dose of HI-6 is correct, but the volume of injection should be 3.05 rather than 3.15 mL.

Impact on Study: Because changes were made prior to the start of the pharmacokinetic study, the solutions prepared should mimic the doses delivered by wet/dry autoinjectors and allow for direct comparison of the different injection techniques.

Carl T. Olson

Carl T. Olson, D.V.M., Ph.D.
Study Director

11-2-90
Date

Don W. Korte, Jr.

LTC Don W. Korte, Jr., M.S.
USAMRICD CUR

2 Nov 90
Date

APPENDIX B

SOPs

Manual Number: MMREF-3.

Battelle SOP Number: TOX VI-014-00
Effective Date: December 28, 1990

Key Words: ATROPINE,
RADIOIMMUNOASSAY, RIA

Standard Operating Procedure (SOP)

THE DETERMINATION OF SERUM ATROPINE SULFATE CONCENTRATIONS BY RADIOIMMUNOASSAY (RIA)

Originated by: Victor S. Moore Date 12-17-90

Approved by: O. M. Kilgore Date 12/20/90
Manager
Toxicology and Pharmacology Dept.

Approved by: Richard A. Lesko Sr. Date 12/21/90
Executive Secretary
Total Quality Council
Health and Environment Group

Reviewed and Registered by QAU: Patricia Reed Date 12-21-90

Distribution List:

Quality Assurance Unit
SOP Manuals

Battelle
Health and Environment Group
505 King Avenue
Columbus, Ohio 43201

I/II. SCOPE/PURPOSE:

The purpose of this Standard Operating Procedure (SOP) is to describe a radioimmunoassay method employed in the determination of serum atropine sulfate concentrations.

III. REFERENCES:

1. Wurzburger, R. J., Miller, R. L., Boxenbaum, H. G., and S. Spector. 1977. Radioimmunoassay of Atropine In Plasma. J Pharmacol Exp Therap 203: 435.

2. Kradjan, W. A., Smallridge, R. C., Davis, R., and P. Verma. 1985. Atropine Serum Concentrations After Multiple Inhaled Doses of Atropine Sulfate. Clin Pharmacol Therap 38: 12.

IV. DEFINITIONS: None

V. PROCEDURES:

Preliminary Tasks

A. Preparation of Phosphate Buffered Saline (PBS), pH 7.5

1. Combine the following components to prepare 1 liter PBS (10 mM Na_2HPO_4, 150 mM NaCl), pH 7.5:

Na_2HPO_4	1.420 grams
NaCl	8.766 grams
distilled water	980.0 ml

2. Adjust the pH to 7.5 with 0.1 N HCl. Bring the volume to 1000 ml with distilled water.

3. Store PBS at 1-9°C. The PBS is stable for a period of one month from the date of preparation.

B. Preparation of Saturated Ammonium Sulfate

1. Combine the following reagents to prepare 500 ml saturated ammonium sulfate:

$(NH_4)_2SO_4$	257.6 grams
distilled water	500.0 ml

2. Do not adjust pH. Store at 1-9°C. This reagent is stable for a period of one month from date of preparation. Prepare at least 24 hours prior to use.

C. Preparation of 50 percent Saturated Ammonium Sulfate

1. Combined the following reagents to prepare 500 ml of 50 percent saturated ammonium sulfate:

$(NH_4)_2SO_4$	128.8 grams
distilled water	500.0 ml

2. Do not adjust pH. Store at 1-9°C. This reagent is stable for a period of one month from the date of preparation. Prepare at least 24 hours prior to use.

D. Preparation of ^{3}H-Atropine Stock Solution

1. ^{3}H-Atropine is prepared in PBS, pH 7.5 at a concentration of approximately 4000 CPM/20 µl. This material is aliquoted and stored at -70 (± 5)°C. The labeled atropine is stable for a period of one year.

2. Thaw a fresh aliquot daily. Dispose of the leftover material at the conclusion of the experiment according to Battelle SOP for disposal of radioactive materials.

E. Preparation of Primary Atropine Stock Solution

1. Prepare a 1.0 mg/ml solution of atropine sulfate in PBS, pH 7.5. Weigh a minimum of 10.0 mg atropine sulfate. Mix thoroughly and aliquot. Store at -70 (± 5)°C. The material is stable for a period of one year from the date of preparation.

F. Preparation of Rabbit Anti-Atropine Antisera Stock

1. The correct concentration of rabbit anti-atropine antisera will be determined in preliminary testing. The stock antisera is stored as 30 µl aliquots at -70 (± 5)°C. Dilute the antisera to the proper concentration in PBS, pH 7.5. Prepare the diluted antibody fresh daily. Leftover material may be frozen and used for repeat analyses performed within a period of five days. Thereafter, dispose of the diluted material.

G. Normal Serum

1. A stock of normal serum obtained from the same species as that of the serum samples being analyzed will be aliquoted and

stored at -70 (± 5)°C. The frozen stock is stable for a period of one year.

2. Aliquot(s) of normal serum are thawed freshly on the assay day. The serum is used undiluted in the assay. Unused material may be frozen and used on a subsequent test day.

H. Test Samples

1. Test samples are stored at - 70 (± 5)°C.

RIA Set Up (Day 1)

1. Prepare atropine sulfate Stocks A and B fresh daily from a freshly thawed aliquot of the Primary Atropine Stock solution as follows:

   a) Combine 10 µl Primary Atropine Stock + 990 µl PBS (Dilution a)
   b) Combine 10 µl Dilution a + 990 µl PBS (Dilution b)
   c) Combine 250 µl Dilution b + 750 µl PBS (Stock A)
   d) Combine 10 µl Dilution b + 990 µl PBS (Stock B)

2. Dispose of the leftover Primary Atropine Stock as well as leftover atropine Stocks A and B and Dilutions a and b at the conclusion of the RIA set up.

3. Prepare Stock C by combining 1.0 ml Stock A with 1.5 ml of normal serum derived from the same species as the sera under analyses. The volumes may be modified proportionately in order to produce the correct volumes for larger or smaller experiments. Dispose of the unused material at the end of the day.

4. Prepare Stock D by combining 200 µl Stock A with 2.3 ml normal sera derived from the same species as the sera under analyses. The volumes may be modified proportionately in order to prepare the correct volumes for larger or smaller experiments. Dispose of the unused material at the end of the test day.

5. The RIA procedure is set up as described on the attached form entitled "Atropine Sulfate Radioimmunoassay Tube Setup". Reagents are aliquoted to 12 x 75 mm polystyrene RIA tubes in order from left to right as indicated in this form.

6. Upon adding all reagents, vortex each tube 5-10 seconds.

7. Incubate the tubes 20 (± 1) hours at 1-9°C.

8. Prepare the total counts control by adding 20 µl ^{3}H-atropine to each of two 20 ml scintillation vials. Add 10.0 ml Hydrofluor and 1.0 ml distilled water to each vial and mix.

Completion of RIA (Day 2)

1. Add 0.5 ml 100 percent saturated ammonium sulfate to each RIA tube. Vortex for 5-10 seconds. Incubate for 30 minutes at 1-9°C. Centrifuge at approximately 2800 RPM (1550 x g) for 30 minutes at room temperature (RT). Carefully aspirate the supernate with a pasteur pipet and transfer the liquid to a container for radioactive liquid waste.

2. Add 1.0 ml 50 percent saturated ammonium sulfate to each tube. Vortex for 5-10 seconds. Centrifuge at approximately 2800 RPM (1550 x g) for 30 minutes at RT. Aspirate the supernate with a pasteur pipet and transfer to a container for radioactive liquid waste.

3. Add 1.0 ml distilled water to each tube to dissolve the pellet. Vortex for 5-10 seconds.

4. Transfer the contents of each RIA tube to a separate scintillation vial by carefully pouring. Rinse the RIA tubes with 2.0 ml Hydrofluor and transfer the fluid to the respective vial.

5. Add 8.0 ml Hydrofluor to each scintillation vial and mix.

6. Count the vials for 10 minutes or to a preset error of 2.0 percent on a liquid scintillation counter.

Data Analysis

1. Data analysis is performed using RiaCalc DM, Version 2.65 (Pharmacia Wallac). Data is reported as ng/ml.

VI. QUALITY CONTROL

1. All equipment and instruments will be operated, calibrated, and maintained according to their respective SOPs.

2. The study director or his designee will review all raw data, completed data forms and other pertinent study records.

3. The form entitled "Atropine Sulfate Radioimmunoassay Tube Setup" details the contents of each standard, control, and sample tube and will be employed daily during assay set up to insure correct distribution of reagents.

4. The form entitled "Record For Instruments, Equipment, Reagents Used For Radioimmunoassay" will be used to document all reagents and equipment used in an assay.

5. The form entitled "Atropine Sulfate RIA Run List" will be utilized to record the identification and assay sequence for controls and samples for an assay.

6. Preparation of buffers and other reagents will be recorded on the attached form entitled "Buffer/Reagent Preparation".

7. A series of low, medium, and high controls are included in each experiment to assess the quality of each experiment. Control data will be tabulated for each run and will be reviewed by the study director.

8. Additional control parameters such as R/T, B/T, the slope and intercept of the regression curve and other parameters are computed by RiaCalc DM. These will be tabulated for each experiment and reviewed by the study director.

BUFFER/REAGENT PREPARATION

Study: _____

Project: _____ Date: _____

Buffer/Reagent: _____

_____

Buffer Storage Conditions: _____ Buffer Expir. Date: _____

Constituents:

Reagent	Supplier	Lot	Receipt Date	Expiration Date	Amount Used

Balance: Description: _____

BCD ID: _____ Location: _____

Standard Weights: BCD ID: _____

Determination No.	Actual Wt.	Wt. Read
1		
2		
3		
4		

pH Adjustment (Reagent and Volume): _____

pH Meter: BCD ID: _____ Final pH: _____

Comments:

Prepared By: _____ Date: _____

Reviewed By: _____ Date: _____

ATROPINE SULFATE RADIOIMMUNOASSAY TUBE SETUP

STUDY CONTROL No _____ PROJECT No.: _____

DATE: _____ RUN No _____ PAGE No: _____

Tube No	Conc.	Standard	Sample	Buffer	Normal Serum	Antibody	^{125}I-Atropine Sulphate
	Standard Curve						
1	T. Tube	None		-			20 uL
2	T. Tube	None					20 uL
3	NSB	None		430 uL	50 uL		20 uL
4	NSB	None		430 uL	50 uL		20 uL
5	0 pg	None		330 uL	50 uL	100 uL	20 uL
6	0 pg	None		330 uL	50 uL	100 uL	20 uL
7	0 pg	None		330 uL	50 uL	100 uL	20 uL
8	0 pg	None		330 uL	50 uL	100 uL	20 uL
9	25 pg	25 uL Stock B		305 uL	50 uL	100 uL	20 uL
10	25 pg	25 uL Stock B		305 uL	50 uL	100 uL	20 uL
11	50 pg	50 uL Stock B		280 uL	50 uL	100 uL	20 uL
12	50 pg	50 uL Stock B		280 uL	50 uL	100 uL	20 uL
13	75 pg	75 uL Stock B		255 uL	50 uL	100 uL	20 uL
14	75 pg	75 uL Stock B		255 uL	50 uL	100 uL	20 uL
15	100 pg	100 uL Stock B		230 uL	50 uL	100 uL	20 uL
16	100 pg	100 uL Stock B		230 uL	50 uL	100 uL	20 uL
17	150 pg	150 uL Stock B		180 uL	50 uL	100 uL	20 uL
18	150 pg	150 uL Stock B		180 uL	50 uL	100 uL	20 uL
19	250 pg	10 uL Stock A		320 uL	50 uL	100 uL	20 uL
20	250 pg	10 uL Stock A		320 uL	50 uL	100 uL	20 uL
21	500 pg	20 uL Stock A		310 uL	50 uL	100 uL	20 uL
22	500 pg	20 uL Stock A		310 uL	50 uL	100 uL	20 uL
23	750 pg	30 uL Stock A		300 uL	50 uL	100 uL	20 uL
24	750 pg	30 uL Stock A		300 uL	50 uL	100 uL	20 uL
25	1000 pg	40 uL Stock A		290 uL	50 uL	100 uL	20 uL
26	1000 pg	40 uL Stock A		290 uL	50 uL	100 uL	20 uL
	Quality Control						
27	100 pg	50 uL Stock D		330 uL		100 uL	20 uL
28	100 pg	50 uL Stock D		330 uL		100 uL	20 uL
29	250 pg	25 uL Stock C		330 uL	25 uL	100 uL	20 uL
30	250 pg	25 uL Stock C		330 uL	25 uL	100 uL	20 uL
31	500 pg	50 uL Stock C		330 uL		100 uL	20 uL
32	500 pg	50 uL Stock C		330 uL		100 uL	20 uL
	Samples (Atropine Run Last)		50 uL	330 uL		100 uL	20 uL
	Samples (Atropine Run Last)		50 uL	330 uL		100 uL	20 uL

Technician Signature: _____ Date: _____

Reviewed By: _____ Date: _____

Atropine Sulfate RIA Run List

(Radioimmunoassay Laboratory)
Battelle, 505 King Avenue, Columbus, OH 43201

Date: ____________ Run No.________ Page No.____
Study Control No:__________ Project No.__________

Tube No.	Sample ID or Code (Source)	Draw Time	Draw Date	Concentration		Comments
				pg/50µL	ng/mL	

Operator Signature: ____________ Date: ____
Reviewed By: ____________ Date: ____

RECORD FOR INSTRUMENTS, EQUIPMENT, REAGENTS USED FOR RADIOIMMUNOASSAY

Project:	Assay:	Project No.
		SC No.

LIST OF INSTRUMENTS/ EQUIPMENT USED

SN	Instrument/ Equipment	Model	Batelle ID	Location
1	Gamma Counter			
2	Scintillation Counter			
3	Water Bath (Temp.)			
4	Heating Blocks/Dry Bath (Temp.)			
5	Incubator (Temp.)			
6	Refrigerator (Temp.)			
7	Freezer (Temp.)			
8				

Other: Incubation Time	In Time:
	Out Time:

LIST OF CHEMICALS, SOLVENTS, AND REAGENTS USED

SN	Name	Cat. #	Lot No.	Exp. Date
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				

Comments:	
Technician Signature:	Date:
Reviewed By:	Date:

STANDARD OPERATING PROCEDURE
MREF SOP-88-31

TITLE: Measurement of Chemical Surety Materiel in Dilute Solutions of GA, GB, GD, TGD, HD-L, HD, L, and VX

LABORATORY: MREF SOP APPROVAL DATE: February 28, 1990

PLACE OF OPERATION OR TEST: Samples throughout MREF; Analyses in Room 17 or Room 37

This Standard Operating Procedure (SOP) has been prepared as prescribed by Contract DAMD17-89-C-9050 and will be effective for one year from date of approval unless sooner rescinded or superseded.

No deviation from this SOP will be permitted. Whenever the approved method is changed, the SOP will be revised.

Supervisory personnel will assure that all personnel involved with this SOP have been properly trained and instructed in its provisions and attest to this requirement by affixing their signatures on page 3.

A copy of this SOP will be posted at the Medical Research and Evaluation Facility (MREF) job site at all times.

Submitted By: Timothy L Haves 2/19/90
Signature/Date

Timothy L. Haves, Principal Research Scientist
Printed Name/Title

Approved By: [signature] 2/22/90
Signature/Date

Garrett S. Dill, D.V.M., Manager
Printed Name/Title

Approved By: [signature] 2/22/90
Signature/Date

David L. Stitcher, CIH, Safety/Surety Officer
Printed Name/Title

Revised February 19, 1990

Approved By:

Sandra M. Anderson 03/07/90
Signature/Date

Quality Assurance Unit
Health and Environment Group
Printed Name/Title

Charles K. Burdick
Signature/Date

Charles K. Burdick, Director
Total Quality Program
Health and Environmental Group
Printed Name/Title

Revised February 19, 1990

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SIGNATURES

I have read and understand the contents of MREF SOP-88-31.

Signature	Date	Signature	Date
Sheri D. Moore	3/30/90		
[illegible]	5/11/90		
[illegible]	5/14/90		
[illegible]	5/23/90		
[illegible]	6-8-90		

Revised February 19, 1990

STANDARD OPERATING PROCEDURE 88-31

Measurement of Chemical Surety Material in Dilute Solutions of GA, GB, GD, TGD, HD-L, HD, L, and VX

A. Statement of Work: This SOP describes analytical methods for the analyses of dilute solutions of chemical surety materiel (CSM) to include dose confirmation samples and dosing stock solutions generated at the MREF. These measurements are performed by comparing the analytical results of exempt chemical surety materiel (XCSM) samples to analytical standards prepared of the same CSM. The analytical standards are prepared and referenced to Standard Analytical Reference Material (SARM) according to MREF SOP-88-30.

The determination of CSM concentration in the diluted samples is performed on a regular basis at the MREF. The analysis must be performed prior to the expiration date established for the particular CSM/solvent combination under the storage conditions described herein. For most program situations, this has been determined to be approximately 2 weeks after sample preparation.

B. Responsibility:

1. Personnel Qualifications: Technical staff will be current with the requirements of the MREF and all applicable MREF SOPs. All technical staff will be familiar with handling hazardous materials within the MREF laboratory. The technical staff must have a fitted SurvivAir respirator in accordance with FSSP SOP-MREF-9. In addition, must know the location of the nerve agent kit, mechanical resuscitator, eye wash fountain, and deluge shower as well as how to use them. They must maintain either visual or audible contact with each other in order to detect unauthorized actions or be ready to rescue or render first aid to the other in the event of an accident. Personnel working with solutions of CSM that do not exceed XCSM criteria must read and sign this SOP.

2. Leaders: Leaders of each operation will be designated by the Study Director for that operation. Each leader will insure that the following are observed:

   a. Only authorized personnel meeting requirements set forth in Section 8.1 are allowed in the room during XCSM operations.

   b. XCSM control and accountability are maintained.

c. Adequate, approved, protective equipment is available at all times to personnel at their work site.

d. All leader and technical staff responsibilities specified in the MREF FSSP are followed.

e. Each employee has been trained in the techniques of administering first aid and self aid.

f. Work under this SOP is performed only in the area(s) or room(s) designated by this SOP.

g. No food, beverage, or tobacco product is consumed, used, or brought into the laboratory. The wearing of contact lenses is prohibited in the laboratory.

h. The safety requirements of this SOP, as well as normal laboratory safety, are maintained.

i. Decontamination solutions are present prior to handling XCSM.

j. All quantities of XCSM that leave the hood or room are properly contained and labeled.

k. All applicable SOPs are read and signed by all technical staff involved in the operation.

3. Technical Staff: Technical staff will be responsible for abiding by requirements set forth in Section B.2. In addition, they must use personal, protective equipment provided and develop safe work habits to protect themselves and fellow workers from injury and to prevent damage to material, equipment, and facilities. They must not perform XCSM operations without the presence of a qualified second person.

4. Research Organization: The organization involved in this research is the MREF of Battelle Memorial Institute, 505 King Avenue, Columbus, Ohio 43201-2693.

C. Materials to be Used:

1. XCSM: XCSM is also referred to as research, development, test, and evaluat on (RDTE) dilute solutions of CSM. The XCSM solutions that can be used following this SOP are those prepared from the following CSM.

a. Tabun (CAS 77-81-6): GA, ethyl N,N-dimethylphosphoramidocyanidate.

b. Sarin (CAS 107-44-8 or 50642-23-4): GB, isopropyl methylphosphonofluoridate.

c. Soman (CAS 96-64-0 or 50642-24-5): GD, pinacolyl methylphosphonofluoridate.

d. VX (CAS 50782-69-9 or 51848-47-6 or 53800-40-1 or 70938-84-0): VX, O-ethyl S-(2-diisopropylaminoethyl)methylphosphonothiolate.

e. Mustard (CAS 505-60-2 or 39472-40-7 or 68157-62-0): HD, bis-dichloroethyl sulfide.

f. Mustard-Lewisite Mixture: HL, a mixture of bis-dichloroethyl sulfide and dichloro(2-chlorovinyl)arsine.

g. Lewisite (CAS 541-25-3): L, dichloro(2-chlorovinyl)arsine.

2. Solvents and Chemicals: Hexane, acetonitrile, or appropriate solvent. Quality of solvent recommended is spectrometric grade, distilled in glass.

3. Decontamination Materials: Sodium hypochlorite (5 percent solution) for XHD, XL, XHDL, and XVX. Sodium hydroxide (10 percent solution) for G agents.

D. Equipment: Safety equipped cart, freezer (locked), refrigerator (locked), latex gloves, labels, first aid kit, plastic-backed, absorbent paper, brown paper, 4-L beakers, squirt bottles, wiping tissues, beakers, bottles, maxi-vials, pipettes, pipette bulbs, tissue paper, laboratory coat, safety shoes, protective eyewear, spatula, stainless-steel pans, bubbler monitors, scissors, solid sorbent traps, glass stir rods, syringes, needles, forceps, GC vials, drierite, 20-mL scintillation vials, an air-supplied respirator with air cylinder, 10-mL volumetric flasks, and a vial support block.

E. Hazards Involved:

1. Anticholinesterase: The hazard from XVX is primarily that of liquid injection, ingestion, or absorption through the skin or eyes. XVX can be lethal if generated in a vapor form in confined or poorly ventilated spaces. Although liquid spills of XVX do not present a vapor hazard, this material is very slow to evaporate so that virtually the entire spill (minus the solvent) may persist as a liquid contact hazard for several days.

a. Mechanism of Action and Physiological Effects: These XCSM cause inhibition of cholinesterase enzyme in the body. Repeated low level exposures to these XCSM will have cumulative effects on

cholinesterase inhibition. Blood cholinesterase is regenerated slowly and the inhibition effect will last several weeks. Clinical signs and symptoms may suddenly occur following repeated exposures, but is unpredictable in time of onset and severity.

Casualty Producing Routes of Entry: Inadvertent skin contact with these XCSM is the most common cause of laboratory accidents/incidents. The XCSM absorption rate will likely be accelerated through unprotected cuts and abrasions.

Signs and Symptoms: The first indication of exposure of anticholinesterase XCSM to the skin is likely to be a reaction at the point of exposure, i.e., localized sweating and/or twitching. If exposed to vapor from some type of vapor generating system, pinpointed pupils (miosis), muscular tightness in the chest, and/or a runny nose will likely be the first symptoms. For other than these extreme exposures, no symptoms are likely to be exhibited. However, under these extreme conditions if the exposure is sufficient, symptoms may progress beyond the local reaction to produce systemic poisoning. The following signs and symptoms are typical of systemic poisoning; the number and severity of which will depend upon degree of exposure:

(1) Nausea--possible vomiting.

(2) Diarrhea.

(3) Weakness.

(4) Muscle twitching.

(5) Convulsions.

(6) Central nervous system depression.

(7) Coma.

(8) Cessation of breathing.

Exposure Factors: Onset of signs and symptoms from a percutaneous exposure may be delayed by the adsorption time. Onset after a vapor inhalation exposure may be quite rapid, and death may occur within 10 min. Vapor exposure to the eyes results in immediate miosis at very low concentrations.

2. Solvents: The solvents used in preparing the dilute material may have hazards associated with their use. A copy of the Material Safety Data

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Sheet (MSDS) is available in the administrative area of the MREF or through Battelle's Safety Office, 505 King Ave.

a. Hexane: Hexane is a flammable liquid that must be handled and stored as a solvent with a dangerous fire risk. The flash point of hexane is -22.7 C, with an autoignition temperature of 260 C. The 1988-1989 American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Values (TLV) for n-hexane is 50 parts per million (ppm) as an 8-hr time weighted average (TWA). For the other hexane isomers, the TLV is 500 ppm as an 8-hr TWA and 1,000 ppm as a 15-min Short Term Exposure Limit (STEL).

b. Acetonitrile: Acetonitrile is a flammable liquid that must be handled as a solvent with a dangerous fire risk. The flash point of acetonitrile is 5.56 C. The 1988-1989 ACGIH TLV for acetonitrile is 40 ppm as an 8-hr TWA and 60 ppm as a 15-min STEL. Also, skin contact may represent a significant route of exposure.

3. Decontamination solutions can cause chemical burns if sodium hydroxide or sodium hypocholride is left in contact with skin or eyes.

4. Gloves and aprons made of butyl rubber are flammable and have no self-extinguishing capability; therefore, care must be taken to avoid open flame or heat that may ignite them.

F. Safety Requirements:

1. Hoods: Hood face velocity must average 100 ± 10 lfpm. The average is computed from individual readings taken in approximately each square foot of hood face (usually nine readings). In addition, no individual reading will vary more than 20 percent from the average. No equipment will be within 20 cm of the face of the hood.

2. Protective Equipment: When working with XCSM samples, the following clothing and protective gear are required as a minimum for all personnel.

lab coat
safety shoes
two pairs of latex gloves
protective eyewear

In addition, each worker's individually assigned Survivair combination escape/airline-supplied respirator will be readily available. All provisions of the MREF FSSP apply to the checking and testing of gloves, aprons, respirators, and other protective equipment.

3. First Aid: A first-aid kit containing two squirt bottles, one filled with a 5 percent available chlorine sodium hypochlorite solution and one filled with water (labeled, dated, and the contents changed every month), and gauze pads will be located in the room. The location of the nearest eye-wash fountain, deluge shower, and fire extinguisher will be known to all workers before work begins.

G. Procedures:

1. Entry: Before entering the secured facility, note the status of the "Agent-in-Use" light at the turnstile. If the "Agent-in-Use" lights are turned on, note the room location and be sure that upon entry to the laboratory area that all safety equipment and procedures described in FSSP SOP MREF-18 are in place. Upon entry of the room, confirm that there are no audible alarms. No operations can be initiated in a room with audible alarms. After entry, personnel will observe the magnehelic gauge on the hood. If inspection reveals that the hood has failed, is marginal in flow, or operates outside the guidelines of FSSP SOP MREF-21, the problem is reported to the MREF Manager and the operation does not begin.

2. Hood Set Up: Prior to obtaining XCSM, the operation hood area must be prepared with all materials necessary to perform an XCSM operation. The hood(s) to be used for any operation with XCSM will contain, as a minimum, the appropriate decontaminating solutions, waste containers, forceps, plastic-backed paper, absorbent tissues, primary container holder, and XCSM transfer equipment. All of the above materials will be kept behind the 8-inch line in the hood.

   Plastic-backed, absorbent paper must be used to protect the work surface of the hood. Five layers of brown paper will be placed on top of the absorbent paper. Two 4-L beakers containing a minimum of 2 L of decontaminating solution will be placed within the hood.

   A vial support block of sufficient size to contain all primary XCSM maxi-vials to be used will be positioned within the hood work area.

3. Handling of XCSM: The handling of XCSM is conducted in accordance with MREF SOP-83-5. The procedures used within this SOP are described in MREF SOP-83-5 and shall include the labeling (Section G.3), obtaining (Section G.4), equilibration (Section G.5), transfer (Section G.6), dilution (Section G.7), transport (Section G.8), packaging (Section G.9), transfer for use within the MREF (Section G.10), and securing of XCSM (Section H).

4. Identification of CSM: All XCSM samples generated must be clearly identified with the following information being regarded as the minimum requirements.

APPROVED

a. Type of XCSM contained in the sample.

b. Solvent used for CSM dilution.

c. Sample preparation date.

d. Sample identification number (e.g., dose confirmation accountability record book number, page number, and sample number and their identifiers as necessary, 50003-03-02 Samp. A1).

e. Estimated analyte concentration based on measured agent purity and dilution procedure.

f. Project or task number under which the sample was prepared.

5. Sample Preparation and Storage: Preparation of samples must be performed using volumetric glassware, pipettes, and/or microsyringes as required to achieve a resulting concentration below agent surety levels (1.0 mg/mL for VX, 2.0 mg/mL for GA, GB and GD, and 10.0 mg/mL for HD). Preparation and handling of dilute samples is performed in accordance with the operational dosing protocol and/or MREF SOP-83-3. An example of the calculations to determine XCSM concentration of samples prepared in accordance with MREF SOP-83-3 is provided below.

Example Calculations:

a. To determine appropriate dilution procedure, consider the required volume of CSM to be delivered and the final volume of the diluent necessary to achieve a resulting expected concentration not greater than the permissable XCSM concentration, see Section G.4. For example, if 10 µL of neat VX is dosed, confirmation of dosing accuracy and syringe precision and accuracy would require delivering the same volume of agent into appropriate volumetric glassware. To determine the appropriate dilution volume, multiply the volume of the CSM to be delivered, in µL, by the density (d = 1.0083 mg/µL at 20 C) of CSM in mg/µL and multiply the multiple by the purity of the CSM used for dilution. The result of this mathematical step is then divided by the target concentration or the maximum XCSM concentration, for VX the value would be 1.0 mg/mL. The following calculation would hold true for this example if the working VX purity is 95 percent:

[(10.0-µL neat VX) (1.0083 mg/µL density of VX)] = 10.083-mg VX
[(10.083-mg VX)(0.95)] = 9.58-mg VX
[9.58-mg VX/10 mL] = .958 mg/mL dilute concentration

Therefore, a volumetric flask of 10 mL or greater would be appropriate for this dilution.

Revised February 19, 1990

b. All dilute solutions should be kept frozen when not in use. All XCSM samples and standard solutions are stored double contained at -70 C in a locked Revco freezer.

6. Standard Preparation: Instrument calibration standards are prepared from standard analytical stock solutions which have been prepared, stored, and referenced to SARM as per MREF SOP-88-30. Calibration standards must be prepared at three concentration levels as a minimum. These concentration levels must extend over the range of expected sample concentration. Appropriate standard concentrations and dilution procedures are to be determined by the chemist at the time of analysis in order to establish precision limits required by sample submitter. The standards will be prepared in the same solvent as the samples unless stability problems in sample preparation solvent have been determined. The recommended solvent for CSM sample preparation for GC analysis is hexane. If standards need to be prepared ahead of time they must be stored at -70 C until analysis.

7. Instrument Set-Up:

a. The GC must be operated with parameters that will yield the best quantitative results for the analytical system. These will vary depending on CSM/solvent combination and concentration levels. The following are recommended as general starting conditions and optimum conditions must be selected by the chemist doing the analyses.

(1) Column - Several analytical columns and detectors have been successfully used to analyze the agents listed in this SOP. Several manufacturers of columns and instruments have also been compared and only minor differences have been observed with all systems evaluated being acceptable under their optimal operating conditions. The recommended column is a general purpose column that produces reliable results with all agents tested to date.

General Analyses:

Column:	30 m x 0.25-mm I.D. SE-54 with .3-μm film thickness
Carrier Gas:	Helium
Velocity :	30 L 5 cm/sec for Helium
Make-up Gas:	30 L 5 mL/min
Detector :	Flame Ionization Detector (FID)
Detector Gases:	H_2 = 400 mL/min L 10 mL/min Air = 40 mL/min L 5 mL/min
Injector Temperature:	275 L 10 C

Revised February 19, 1990

APPROVED

Detector Temperature:	250 L 10 C
Oven Program:	Initial temperature = 60 C Initial time = 1.0 min Level 1 program rate = 15 C/min Final temperature = 250 C Final time = 2.0 min Post value = 275 C Post time = 4.0 min
Injection Mode:	Split
Split Flow:	120 L 10 mL/min
Split Liner Packing:	3 percent OV-1 on 80/100 mesh Chromosorb WHP (2-3 mm bed)
Injection Volume:	1 μL
Auto Sampler:	Hewlett Packard 7673A or equivalent with cooled sample tray maintained at 5-7 C.

Analysis of Samples of GD in 0.9 percent Biological Saline:

Column:	25 m x 0.32-mm I.D. HP-20 M with .3-μm film thickness or equivalent
Carrier Gas:	Helium
Velocity :	30 L 5 cm/sec for Helium
Make-up Gas:	30 L 5 mL/min
Detector :	FPD with 525-nm phosphorous selective filter
Detector Gases:	H_2 = 135 mL/min L 10 mL/min Air = 120 mL/min L 5 mL/min O_2 = 15 mL/min L 2 mL/min
Injector Temperature:	140 L 5 C (Very important for satisfactory precision and accuracy of results.)
Detector Temperature:	225 L 5 C
Oven Program:	Initial temperature = 50 C Initial time = 0.5 min Level 1 program rate = 20 C/min Final temperature = 200 C Final time = 1.0 min Post value = 215 C Post time = 2.0 min

Analysis of Samples of GA in Multisol:

Column:	25 m x 0.32-mm I.D. HP-20M with .3-μm film thickness or equivalent
Carrier Gas:	Helium
Velocity :	30 L 5 cm/sec for Helium
Make-up Gas:	30 L 5 mL/min
Detector :	FPD with 525-nm phosphorous selective filter
Detector Gases:	H_2 = 135 mL/min L 10 mL/min Air = 120 mL/min L 5 mL/min

Revised February 19, 1990

APPROVED

	O_2 = 15 mL/min L 2 mL/min
Injector Temperature:	140 L 5 C (Very important for satisfactory precision and accuracy of results.)
Detector Temperature:	225 L 5 C
Oven Program:	Initial temperature = 80 C Initial time = 0.5 min Level 1 program rate = 20 C/min Final temperature = 200 C Final time = 1.0 min Post value = 215 C Post time = 2.0 min
Injection Mode:	Split
Split Flow:	120 L 10 mL/min
Split Liner Packing:	10 percent OV-1 on 80/100 mesh Chromosorb WHP (2-3 mm bed)
Injection Volume:	1 µL
Auto Sampler:	Hewlett Packard 7673A or equivalent with cooled sample tray maintained at 5-7 C.

Note: The viscosity of multisol prohibits reproducible injections of samples using an autosampler. Therefore, samples in multisol have to be diluted with an appropriate solvent such as tetrahydrofuran (THF). The samples have been shown to be stable for at least 72 hr after dilution at 5-7 C. The recommended dilution is a minimum factor of five for reliable injection results using an autosampler. As previously stated, the standards should also be diluted using THF.

Analysis of TGD Samples in Acetonitrile:

Column:	25 m x 0.32-mm I.D. HP-20M with .3-µm film thickness or equivalent
Carrier Gas:	Helium
Velocity :	30 L 5 cm/sec for Helium
Make-up Gas:	30 L 5 mL/min
Detector :	FPD with 525-nm phosphorous selective filter
Detector Gases:	H_2 = 135 mL/min L 10 mL/min Air = 120 mL/min L 5 mL/min O_2 = 15 mL/min L 2 mL/min
Injector Temperature:	200 L 5 C (Very important for satisfactory precision and accuracy of results.)
Detector Temperature:	225 L 5 C
Oven Program:	Initial temperature = 50 C Initial time = 0.5 min Level 1 program rate = 20 C/min Final temperature = 200 C Final time = 1.0 min

Revised February 19, 1990

Post value = 215 C
Post time = 2.0 min

Injection Mode:	Split for concentrations above 100 µg/mL Splitless for concentrations below 100 µg/mL
Split Flow:	120 L 10 mL/min
Split Liner Packing:	10 percent OV-1 on 80/100 mesh Chromosorb WHP (2-3 mm bed)
Injection Volume:	1 µL
Auto Sampler:	Hewlett Packard 7673A or equivalent with cooled sample tray maintained at 5-7 C.

Note: Acetonitrile has been selected for dilution of neat TGD due to increased solubility of thickener and agent stability in this solvent.

b. Install the proper column into the capillary injector and detector ports and leak test the joints. If the column has not been in use, condition at 20 C below manufacturer suggested maximum operation temperature overnight. This must be done by first allowing the column to set at room temperature with carrier gas flow for ≈ 20 min and then programming to the final temperature at a slow rate such as 3 C/min. The column can then be left at upper temperature overnight. This initial conditioning is required to insure that oxidants trapped in the column under storage conditions are removed prior to exposure of the column to elevated temperatures. The exposure of the capillary column liquid support to elevated temperatures in the presence of compounds such as oxygen or water will decompose the stationary phase of a capillary column. The exposure of fused silica, the material of which most modern columns are prepared, to water will dissolve the material and also destroy the column by producing chemically bonding active sites within the column.

c. Set the temperatures in the heated zones using the GC terminal using the appropriate example temperatures as listed in Section G.6.a.(1).

d. Set the gas flow rates as recommended in Section G.6.a.(1) using a soap bubble flow meter of appropriate range and stopwatch. Set the carrier velocity first then turn the detector gases off. The column velocity is measured by injecting a compound under conditions that will yield an insignificant retention of the compound. A typical compound used to set column velocities is methane for FID conditions or acetone for FPD. The column velocity is measured by injecting the methane and timing the time required for the methane or acetone to exit the column. The exit of the methane or acetone is monitored by the detector, and when

the peak is observed, the time is recorded. A sample calculation is:

$$\frac{\text{Column Length in cm}}{\text{Retention Time of Methane or Acetone in sec}} = \text{Carrier Velocity in cm/sec}$$

The column velocity must be set at optimum column temperature (mid-range of the operating conditions) since capillary column velocities change with temperature. The recommended temperature for the recommended conditions is 150 C. Once column velocity has been set, allow the carrier gas to flow continuously through the column during the remainder of the measurements. It should be noted if a carrier gas other than helium is used, a Van Deemter Curve for that gas should be reviewed to establish the optimum velocity.

e. Attach the flow meter to the gas outlet from the detector with the carrier gas on and all other gases off. Measure the column flow rate and record as this will need to be subtracted from all future measurements to get actual flow rates.

Example Calculation for Flow Rate:

(i.e., for a time interval of 15 sec to achieve a volume of 10 mL the following calculation would be performed)

Formula for determining flow rate:

$$\text{Flow rate in mL/min} = \frac{\text{Volume (mL)}}{\text{Time (min)}}$$

$$\frac{15 \text{ sec}}{60 \text{ sec/min}} = .25 \text{ min}$$

$$\frac{10 \text{ mL}}{.25 \text{ min}} = 40 \text{ mL/min (flow rate)}$$

f. Adjust and repeat measurements until the prescribed value is reached subtracting the carrier flow rate from the observed flow rate to get actual flow rates.

g. Repeat measurement procedure to set hydrogen flow rate.

h. Repeat measurement procedure to set air flow rate.

i. With gases on, ignite the FID or FPD flame by depressing the flame ignition button. Verify flame ignition by checking for continuous

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condensation on a cold surface (e.g., mirror) at the effluent outlet on the FID or FPD.

j. When the flame has been ignited, turn on the FID or FPD electronics and allow 20 min for system equilibration.

k. Key in the following set points on the integrator terminal if available. If a strip chart is used, refer to instrument manual for connections. Typical settings are:

1. Integration Method: Area percent

2. Attenuation: $(2)^3$

3. Percent Offset: 10

4. Peak Width: 0.04

5. Threshold: 4

6. Run Time: 15.0 STOP

7. Chart Speed: 0.5

8. Detector: on

These set points are only guidelines, but entering values for these parameters is a minimum requirement for integration.

l. Plot the FID or FPD signal on the GC recorder. Zero the plot on the terminal or strip chart recorder so that the baseline is at ≈ 10 percent offset.

8. Analysis of Samples: Standards and sample solutions are analyzed using the same procedures.

9. Calculation Procedures:

a. Identify the C. peak in the sample and standard chromatograms; record the peak area.

b. To calculate the concentration of the XCSM samples, construct a calibration curve by doing a linear regression of standard concentration vs. standard peak area for all concentration levels, then fit the sample peak area to the curve to obtain concentration.

10. Quality Control:

APPROVED

a. Each step in the analysis of standards and samples must be done reproducibly to achieve good precision and accuracy. This includes preparation of dilute solutions and instrument operation.

b. The samples are to be injected a minimum of three times each with an average response used to determine the purity measurement. The relative standard deviation for any set of injections must not exceed 10 percent. If the relative standard deviation exceeds 10 percent, the experiment must be repeated entirely prior to acceptance of data.

c. The FID is a general purpose GC detector. The detector is linear over an extremely large range which makes it well suited for this type of analysis. In addition, the detector is general purpose in that it detects almost all chromatographable material with two or more carbon atoms. However, due to the non-selective nature of the detector, the detection of interferences may be encountered. Therefore, each new type of testing should be preceded by experimentation to determine whether any interferences are present and if so, to identify and compensate for them.

d. Blanks for solvents must be checked and high purity solvents such as distilled in glass are recommended. An analysis of the system blank must be studied under the same test conditions (first without CSM present and then with CSM spike) and compare the chromatograms. Evaluate the data and make any necessary corrections.

e. If interferences present a problem, then analysis using a mass spectrometer for the detection system is recommended so that interferences can be confirmed and possibly identified as solvent impurities or CSM impurities. A mass spectrometer should also be used when initially establishing GC conditions to insure that the chromatographic peak being measured during purity analyses is the CSM of interest and not an impurity.

11. Instrument Shut-Down:

a. When the instrument is not to be used for extended periods of time, the system must be shut down following manufacturer's instructions to insure column life and instrument stability.

b. Be sure that sufficient gases are supplied for continuous flow of carrier and detector gases for the period of time that the system will be unattended.

APPROVED

Revised February 19, 1990

c. For weekend shut-down, follow the same procedure but also extinguish the detector flame if appropriate by shutting off the detector gas valves.

H. Decontamination: Proper protective equipment and clothing must be utilized throughout these operations in accordance with FSSP SOPs MREF-23 and/or MREF-25. All absorbent material covering the hood surface will be placed in the decontaminating solution after each operation. All disposable glassware in hoods will be submerged in the appropriate decontaminating solution (5 percent available chlorine in a sodium hypochlorite solution or 10 percent sodium hydroxide solution in water) overnight. All non-disposable glassware in hoods will be filled with the appropriate decontaminating solution (5 percent available chlorine in a sodium hypochlorite solution or 10 percent sodium hydroxide solution in water) overnight. Materials left to soak in decontaminating solution overnight will be removed from the hood on the next work day. The glassware, equipment, or non-expendable materials are rinsed with water and removed from the hood. Expendable items may be placed directly into a primary plastic bag within the hood. The primary plastic bag is then sealed with adhesive tape and placed inside another plastic bag, which is then sealed with adhesive tape to provide double containment of decontaminated materials.

Bags of waste must be labeled "Contaminated Materials" with type of XCSM, date of bagging, bag identification number, and name of person packaging the contaminated materials in accordance with MREF SOP-83-3, Section H. The double-contained materials can then be incinerated.

I. Emergency Procedures: If an XCSM spill occurs, decontamination solution (containing 5 percent sodium hypochlorite or 10 percent sodium hydroxide) located within the hood is gently poured or swabbed with soaked absorbent paper held with forceps on the area in an amount that is at least tenfold in excess of the spill. This contaminated decontaminating solution is absorbed with diatomaceous earth or other absorbent and deposited into double plastic bags. The cleaning/absorption procedure is repeated again.

In the event of any incident or exposure, the MREF Manager or his designee must be notified immediately.

J. First Aid Procedures: Make sure that you protect yourself from contamination by the casualty. Mask if in doubt. Personnel exposed to a toxic agent will be removed immediately to a shower area where washing and first aid can be administered by co-workers. If there is any question about the source of contamination, place the victim under the emergency shower. Wash the victim down with soap; do not scrub as this may enhance penetration.

1. Emergency Treatment for Specific Types of XCSM:

   a. V and G XCSM:

      (1) Decontaminate when the source of contact is certain.

         (a) Transfer the victim to a clean area and thoroughly decontaminate with 5 percent sodium hypochlorite only in the areas below the eyes in the position in which the victim is being held. Wash skin at once with diluted chlorine-type bleach and rinse with copious amounts of water. Rinse eyes with water only; rinsing a minimum of 10 min at the eyewash fountain. Decontaminate with dry tissue or absorbent paper followed by water in areas close to the eyes.

      (2) Decontaminate when source of contact is uncertain.

         (a) Place victim in shower and remove clothing.

      (3) If victim has symptoms of anticholinesterase poisoning beyond miosis, inject him with the contents of the atropine/2-PAM autoinjectors at intervals of 5-10 min up to a maximum of three injections. Note time of each injection on the victim for reference by physician.

      (4) If victim has stopped breathing, employ resuscitation with the ambu-bag immediately. Use the atropine autoinjectors after you have successfully succeeded in restoring respiration.

   b. H and L XCSM:

      (1) Decontamination when the source is certain.

         (a) Transfer the victim to a clean area and thoroughly decontaminate with 5 percent sodium hypochlorite only in the areas below the eyes in the position in which the victim is being held. Wash skin at once with diluted chlorine-type bleach and rinse with copious amounts of water. Rinse eyes with water only; rinsing a minimum of 10 min at the eyewash fountain. Decontaminate with dry tissue or absorbent paper followed by water in areas close to the eyes.

      (2) Place victim in shower and remove clothing.

2. The decontaminated individual is transported by ambulance to University Hospital.

3. In the event of any exposure, the MREF Manager or his designee must be summoned after the immediate emergency is taken care of and informed of the exposure.

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Revised February 19, 1990

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STANDARD OPERATING PROCEDURE
MREF SOP-88-46

TITLE: Measurement of the Ability of Chemicals to Decrease the Aging Rate of Soman (GD)-Inhibited Acetylcholinesterase (AChE) Activity, In Vitro

LABORATORY: MREF SOP APPROVAL DATE: April 18, 1990

PLACE OF OPERATION OR TEST: Rooms 2, 3, 5, 7, 47, and 47A

This Standard Operating Procedure (SOP) has been prepared as prescribed by Contract DAMD17-89-C-9050 and will be effective for one year from date of approval unless rescinded or superseded.

No deviation from this SOP will be permitted. Whenever the approved method is changed, the SOP will be revised.

Supervisory personnel will assure that all personnel involved with this SOP have been properly trained and instructed in its provisions and attest to this requirement by affixing their signatures on page 3.

A copy of this SOP will be posted at the Medical Research and Evaluati Facility (MREF) job site at all times.

Submitted By: David W. Hobson 4/13/90
Signature/Date

David W. Hobson, Ph.D., Associate Manager
Printed Name/Title

Approved By: 4/13/90
Signature/Date

Garrett S. Dill, D.V.M., Manag
Printed Name/Title

Approved By: 4/18/90
Signature/Date

David L. Stitcher, CIH, Safety/Surety Officer
Printed Name/Title

Revised April 11, 1990

Approved By:

Linda M. Anderson 04/19/90
Signature/Date

Quality Assurance Unit
Health and Environment Group
Printed Name/Title

Chas K. Burdick 4/23/90
Signature/Date

Charles K. Burdick, Director
Total Quality Program
Health and Environment Group
Printed Name/Title

Revised April 11, 1990

APPROVE

SIGNATURES

I have read and understand the contents of MREF SOP-88-46.

Signature	Date	Signature	Date
[signature]	05-90		
[signature]	5-3-90		
[signature]	5-3-90		
[signature]	5/9/90		
Cindy Pelley	051790		
[signature]	5/18/90		

STANDARD OPERATING PROCEDURE 88-46

Measurement of the Ability of Chemicals to Decrease the Aging Rate of Soman (GD)-Inhibited Acetylcholinesterase (AChE) Activity, In Vitro

A. Statement of Work: Acute toxicity associated with organophosphates has been attributed to inhibition of acetylcholinesterase (AChE) which causes the muscarinic, nicotinic, and central nervous system cholinergic effects observed with organophosphate (OP) exposure. There are marked differences amongst the various OPs in the persistence of AChE inhibition. In this SOP, an assay is outlined for the use of an in vitro system to identify candidate pretreatment and treatment (P&T) compounds that significantly decrease the aging rate of Soman (GD)-inhibited AChE (GD-AChE).

B. Responsibility:

1. Personnel Qualifications: Technical staff will be current with the requirements of the MREF and all applicable MREF SOPs. All technical staff will be familiar with handling hazardous materials within the MREF laboratory. The technical staff must have a fitted SurvivAir respirator in accordance with FSSP SOP MREF-9. They must know the location of the nerve agent kit, mechanical resuscitator, eye wash fountain, and deluge shower as well as how to use them. They must maintain either visual or audible contact with each other in order to detect unauthorized actions or be ready to rescue or render first aid to the other in the event of an accident. Personnel working with solutions of chemical surety materiel (CSM) that do not exceed XCSM criteria must read and sign this SOP before performing the procedure.

2. Leaders: Leaders of each operation will be designated by the Study Director for that operation. Each leader will insure that the following are observed:

a. Only authorized personnel meeting requirements set forth in Section B.1 are allowed in the room during XCSM operations.

b. XCSM control and accountability are maintained.

c. Adequate, approved, protective equipment is available at all times to personnel at their work site.

d. All leader and technical staff responsibilities specified in the MREF FSSP are followed.

Revised April 11, 1990

e. Each employee has been trained in the techniques of administering first aid and self aid.

f. Work under this SOP is performed only in the area(s) or room(s) designated by this SOP.

g. No food, beverage, or tobacco product is consumed, used, or brought into the laboratory. The wearing of contact lenses is prohibited in the laboratory.

h. The safety requirements of this SOP, as well as normal laboratory safety, are maintained.

i. Decontamination solutions are present prior to handling XCSMs.

j. All quantities of XCSM that leave the hood or room are properly contained and labeled.

k. All applicable SOPs are read and signed by all technical staff involved in the operation.

3. Technical Staff: Technical staff will be responsible for abiding by requirements set forth in Section B.2. In addition, they must use personal, protective equipment provided and develop safe work habits to protect themselves and fellow workers from injury and to prevent damage to material, equipment, and facilities. They must not perform XCSM operations without the presence of a qualified second person.

4. Research Organization: The organization involved in this research is the MREF of Battelle Memorial Institute, 505 King Avenue, Columbus, Ohio 43201-2693.

C. Materials to be Used:

1. XCSM: XCSM is also referred to as research, development, test, and evaluation (RDTE) dilute solutions of CSM. The XCSM solutions that can be used following this SOP are those prepared from the following CSM.

a. Soman (CAS 96-64-0 or 50642-24-5): GD, pinacolyl methylphosphonofluoridate.

2. Decontamination Materials: Sodium hydroxide (NaOH) (10 percent in solution).

Revised April 11, 1990

APPROVED

D. Equipment: Freezer and refrigerator; volume-adjustable single and multichannel pipettors; pipette tips; pipette-aid and serological pipettes; COBAS/FARA Centrifugal Analyzer; 96-well, flat-bottomed microtiter plates; microtiter plate reader; 10-mL and 25-µL volumetric flasks; 20-mL scintillation vials; plastic-backed, absorbent paper; absorbent wipes (e.g. Kimwipes); 4-L container; two 500-mL squirt bottles; solid adsorbent material; plastic bags; atropine/pralidoxime chloride (2-PAM) autoinjectors; and first aid kit.

E. Hazards Involved:

1. Anticholinesterase: The hazard from XGD is primarily that of liquid injection, ingestion, or absorption through the skin or eyes.

a. Mechanism of Action and Physiological Effects: This XCSM causes inhibition of AChE in the body. Repeated low level exposures to this XCSM will have cumulative effects on cholinesterase inhibition. Blood AChE is regenerated slowly, and the inhibition effect will last several weeks. Clinical signs and symptoms may suddenly occur following repeated exposures, but is unpredictable in time of onset and severity.

Inadvertent skin contact with XCSM is the most common cause of laboratory accidents/ incidents. The XCSM absorption rate will likely be accelerated through unprotected cuts and abrasions. The first indication of exposure of skin to XGD is likely to be a reaction at the point of exposure, i.e., localized sweating and/or twitching. If exposed to vapor pinpoint pupils (miosis), muscular tightness in the chest, and/or a runny nose will likely be the first effects. Other than the above, no symptoms are likely to be exhibited from exposure to XGD. However, under extreme and unusual circumstances an exposure may be sufficient such that symptoms may progress beyond a local reaction to produce systemic poisoning. The following signs and symptoms, the number and severity of which will depend upon degree of exposure, are typical of systemic toxicosis:

(1) Nausea--possible vomiting.

(2) Diarrhea.

(3) Weakness.

(4) Muscle twitching.

(5) Convulsions.

Revised April 11, 1990

(6) Central nervous system depression.

(7) Coma.

(8) Cessation of breathing.

(9) Exposure Factors: Onset of signs and symptoms from a percutaneous exposure may be delayed by the absorption time. Onset after a vapor inhalation exposure may be quite rapid, and death may occur within 10 min. Vapor exposure to the eyes results in immediate miosis at very low concentrations.

2. Decontamination solutions can cause chemical burns if NaOH is left in contact with skin or eyes.

3. Gloves and aprons made of butyl rubber are flammable and have no self-extinguishing capability; therefore, care must be taken to avoid open flame or heat that may ignite them.

F. Safety Requirements:

1. Hoods: Hood face velocity must average 100 ± 10 lfpm. The average is computed from individual readings taken in approximately each square foot of hood face (usually nine readings). In addition, no individual reading will vary more than 20 percent from the average. No equipment will be within 20 cm of the face of the hood.

2. Protective Equipment: When working with XCSM samples, the following clothing and protective gear are required as a minimum for all personnel.

lab coat
safety shoes
two pairs of latex gloves
protective eyewear

In addition, each worker's individually assigned Survivair combination escape/airline-supplied respirator will be readily available. All provisions of the MREF FSSP apply to the checking and testing of gloves, aprons, respirators, and other protective equipment.

3. First Aid: A first aid kit containing two squirt bottles, one filled with a 5 percent available chlorine NaOCl solution and one filled with water (labeled, dated, and the contents changed every month), and gauze pads will be located in the room. The location of the nearest

eye-wash fountain, deluge shower, and fire extinguisher will be known to all laboratory personnel before work begins.

G. Experimental System: This assay entails the development, validation, and use of an in vitro assay to identify candidate compounds that decrease the rate of aging of GD-inhibited AChE (GD-AChE).

H. Experimental Design: Using standardized methodology, automated as necessary to permit the screening of at least 12 P&T compounds per week, the effect of each compound is evaluated relative to its ability to inhibit, in vitro, the aging rate of GD on AChE. The procedure is based on the procedure described by Puu et al., and involves determining the amount of reactivatible GD-inhibited fetal bovine serum (FBS) AChE as a function of GD incubation time. For general screening, a minimum of two time points are required, however, additional time points may be estimated if mandated by experimental protocol.

I. Procedures:

1. Hood Set-Up: Prior to obtaining XCSM, the operation hood area must be prepared with all materials necessary to perform an XCSM operation. The hood(s) to be used for any operation with XCSM will contain, as a minimum, the appropriate decontaminating solutions, waste containers, forceps, plastic-backed absorbent paper, primary container holder, and XCSM transfer equipment. All of the above materials will be kept behind the 8-inch line in the hood.

   Plastic-backed absorbent paper must be used to protect the work surface of the hood. Five layers of brown paper will be placed on top of the absorbent paper. Two 4-L beakers containing a minimum of 2 L of decontaminating solution will be placed within the hood.

   A vial support block of sufficient size to contain all primary XCSM maxi-vials to be used will be positioned within the hood work area.

2. Preparation of AChE Assay Reagents:

   a. Phosphate Buffer (0.1 M, pH 7.6): This reagent has an expiration date of 8 weeks following the date of preparation.

      Solution A: Dissolve 1.38 of monobasic sodium phosphate in 50-mL deionized water.

      Solution B: Dissolve 7.1 g of dibasic sodium phosphate in 250-mL deionized water.

APPRO

Mix 32.5 mL of Solution A with 217.5 mL of Solution B in a 500-mL volumetric flask. Bring total volume to 500 mL with deionized water. Adjust to pH 7.6 as necessary using 1.0 N HCl or 1.0 M NaOH.

b. TRIS(hydroxymethyl)-aminomethane (TRIS) Buffer: This reagent has an expiration date of 8 weeks following the date of preparation.

(1) COBAS Assay - 0.05 M, pH 8.2: Dissolve 3.03 g of TRIS in 400-mL deionized water and adjust to pH with 6.0 N HCl. Bring to 500 mL with deionized water.

(2) Microplate Assay - 0.1 M, pH 8.2: Dissolve 6.06 g of TRIS in 400 mL of deionized water and adjust to pH with 6.0 N HCl. Bring to 500 mL with deionized water.

c. 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) Reagent: This reagent is not used 4 weeks after the date of preparation.

(1) COBAS Assay - (0.42 mM): Dissolve 0.0333 g DTNB in approximately 150-mL TRIS buffer. Adjust pH to 8.2 with 1.0 M NaOH. Bring to a total volume of 200 mL with deionized water.

(2) Microplate Assay - (0.84 mM): Dissolve 0.0666 g of DTNB in approximately 150-mL TRIS buffer. Adjust pH to 8.2 with 1.0 M NaOH. Bring to a total volume of 200 mL with deionized water.

d. Saline [0.9 Percent Sodium Chloride (NaCl)]: Dissolve 0.9 g NaCl in 90-mL deionized water. Bring to a final volume of 100 mL with deionized water. This reagent is not used 8 weeks after the date of preparation.

e. Acetylthiocholine Iodine (ATCHI) Substrate (5×10^{-2} M): Dissolve 5.784-g ATCHI in 90-mL saline. Dilute to a final volume of 100 mL with saline. Aliquot into 2.5 mL in-use quantities, label, and store at 0 to -20 C. This reagent is not used 6 months following the date of preparation.

(1) COBAS Assay - Dilute 1:4 with saline before use.

(2) Microplate Assay - Dilute 1:15 with saline before use.

f. Assay Buffer: To 100 mL of phosphate buffer (a), add 0.20-g magnesium chloride ($MgCl_2$) (0.01 M), 0.01-g bovine serum albumin.

Revised April 11, 1990

APPROVED

and 0.002-g sodium azide (0.002 percent). Store refrigerated until use. This reagent may be used up to 4 weeks past the date of preparation.

g. AChE Stock Solution (EC 3.1.1.7): Either FBS AChE or eel AChE may be used as specified by the experimental protocol. FBS AChE is obtained from the Department of Biochemistry, Walter Reed Army Institute of Research. It is supplied at a concentration of approximately 5,000 U/mL in 10 percent glycerol and 0.1 M sodium phosphate buffer (pH 7.6) in pre-assayed vials and may be stored for at least a year at -10 C with less than 10 percent activity loss. (NOTE: The storage temperature must not ever be lower than -25 C.) Eel AChE is purchased commercially as a lyophilized powder of approximately 300.0 U/mg protein. This powder is diluted to produce an AChE stock solution with a concentration of approximately 5,000 U/mL by the addition of 5,000 U of the powder to each 1.0 mL of a 10 percent glycerol and 0.1 M sodium phosphate buffer (pH 7.6) solution which is subsequently aliquoted into vials containing 0.5 mL each and stored at -10 C until use. The aliquots used are not restored for next day of later use. Frozen aliquots may be used for up to 1 year past the date of preparation.

h. AChE Reagent Solution:

(1) COBAS Assay: Add 250 µL of packed erythrocyte sample in a microcentrifuge tube. Dilute to 1 mL with deionized water and mix to hemolyze the erythrocytes. Centrifuge for 10 min at 12,000 to 15,000 revolutions per min (rpm) in a Brinkman microcentrifuge. Decant the supernate and add 1 mL of deionized water to the pellet. Resuspend the pellet and centrifuge as before. Decant the second supernate and resuspend the pellet in approximately 1.0 mL of 1 percent triton X-100 in normal saline. The volume in which the pellet is resuspended may fluctuate depending on the level of red cell AChE. For Rhesus monkey erythrocytes, 1.0 mL is a good starting volume. Assay the solubilized material for AChE activity using the COBAS/FARA analyzer using the GDR program. Then dilute the material to the desired AChE activity, for use in the GD aging assay, with the 1 percent triton X-100 solution. If the amount of red cell membrane AChE is low, a larger starting volume of packed red blood cells may be used. FBS AChE: This is used for testing the effect of P&T compounds on AChE aging of GD-inhibited enzyme. Dilute the AChE stock as in Section I.1.g above with assay

buffer to make a solution that has an approximate AChE activity of 2 U/mL.

(2) Microplate Assay: Dilute the AChE stock solution described in Section I.1.g above with assay buffer to make a solution that has an approximate AChE activity content of 1.0 U/mL.

i. HI-6 Preparation (2.8 mM): A 2.8 mM solution is prepared by dissolving 24.9 mg of HI-6 in approximately 15 mL of assay buffer in a 25-mL volumetric flask. The pH is adjusted to 7.6 and the volume is then brought to 25 mL with assay buffer. The final incubation concentration of reactivator should be 800 μM. As indicated by protocol, 2-PAM or other reactivators may be used in place of HI-6.

j. P&T Compound Stock Solutions (2.0 mM): Add 50.0 μmol of each P&T compound to approximately 15-mL assay buffer in a 25-mL volumetric flask. Mix and bring to a final volume of 25 mL with assay buffer. Adjust the pH to 7.6 as needed.

3. Handling of XCSM: The handling of XCSM is conducted in accordance with MREF SOP-83-5. The procedures used within this SOP are described in MREF SOP-83-5 and shall include the labeling (Section G.3), obtaining (Section G.4), equilibration (Section G.5), transfer (Section G.6), dilution (Section G.7), transport (Section G.8), packaging (Section G.9), transfer for use within the MREF (Section G.10), and securing of XCSM (Section H).

4. Dilution of GD to XCSM Levels:

a. GD is diluted to XCSM levels (0.5 mg/mL) in MREF Room 2B following the procedure set forth in FSSP SOP MREF-10 and MREF SOP-83-3. The preparation of dilutions and the transfer of XCSM is recorded in a logbook to document each step of operation and to create a permanent record.

b. The diluent for GD is distilled and/or deionized water. The primary container for the XGD is a 1.0 mL septum-capped vial, clearly labeled to indicate the contents, dilution, date of preparation, and the name of the individual preparing the dilution. GD dilutions are stored between -60 to -80 C until used. XGD, prepared and stored in this fashion, will not be used 90 days past the date of preparation.

c. For routine assay use, a vial is removed from storage and is allowed to thaw in an XCSM-approved laboratory hood or biological safety cabinet.

(1) COBAS Assay: The XGD is prepared in assay buffer and the concentrations used may fluctuate depending on the source of the cholinesterase and degree of inhibition desired. For Rhesus monkey AChE samples, 0.5 to 5 nM XGD will inhibit the enzyme approximately 25 to 75 percent, respectively, under the above incubation conditions. For the testing of the effect of P&T compounds on the aging rate use the same XGD concentration as in the microplate assay.

(2) Microplate Assay: A working dilution is then prepared by further dilution of the vial contents to a concentration of approximately 0.05 μM. This working solution is kept in a sealed container on ice until used in the assay.

5. Storage of XCSM:

a. XCSM is stored according to the procedures set forth in MREF SOP-83-5. All exempt level protective equipment is worn during the transport and storage procedure.

b. The primary container in the secondary container is stored in the freezer in Room 47A, which is secured by locking the door with an approved combination lock and hasp.

6. Microplate Assay Procedure:

a. The microtiter plate reader is turned on and programmed to read absorbance at 414 nm for up to a 5 min period. Recording of change in absorbance data as a function of time is accomplished either by use of the printer on the microtiter plate reader or via electronic data capture using a computer connected to the built-in data interface on the microtiter plate reader.

b. Using the reagents prepared as described in Section I.1 and multichannel pipettors (4, 8, 12, or 96 channel, 5-150 μL), 96-well flat bottom microtiter plates (8 rows x 12 columns) are prepared for each AChE assay.

- Initialize each microtiter plate by collection of a blank reading against air.

APPRO

- AChE Controls - Pipette 10 µL of assay buffer into each well of the plate (a minimum of 4 wells is required for each aging time point to be estimated). Add 20 µL of AChE assay solution and incubate at room temperature for the specified aging interval (to simulate aging time). After a minimum of 15 sec, add 20 µL of assay buffer. Following incubation for the aging interval, add 20 µL of assay buffer or 2.8 mM HI-6 and incubate at room temperature for 5 min, then assay for AChE activity.

- GD-Inhibited AChE Controls - Pipette 10 µL of XCSM GD solution into each well of the plate (minimum 4 wells for each aging time). Add 20 µL of AChE assay solution at time 0 and 20 µL of assay buffer and incubate for at least 15 sec. Incubate for the specified aging time, add 20 µL of HI-6 solution, then incubate for 5 min and assay for AChE activity.

- Reactivator Controls - Pipette 10 µL of assay buffer into each well of the plate. Add 20 µL of AChE, after at least 15 sec, add assay solution. Add 20 µL of AChE assay buffer into each well (a minimum of 4 wells for each P&T compound concentration and aging time combination). Incubate for the specific aging interval to simulate AChE aging time, then add 20 µL of the HI-6 solution. Incubate for 5 min and assay for AChE activity.

- Reactivation Samples - Pipette 10 µL of XCSM GD solution into each well (a minimum of 4 wells per each reactivator concentration and aging time combination). Add 20 µL of AChE assay solution and incubate for at least 15 sec. Add 20 µL of a candidate P&T solution and, after the designated aging time, add 20 µL of HI-6 solution. Incubate for 5 min and assay for AChE activity.

c. The AChE-GD aging time permits can be as short as 30 sec. The aging periods used is dependent upon the aging rate of GD-inhibited AChE. This may vary between species.

d. Assay for AChE Activity: Following incubation, add 75 µL of 0.84 mM DTNB solution to each well and mix for 30 sec. When erythrocyte AChE preparations are used, this time interval should be increased to 90 sec. ATCHI substrate (75 µL) solution is then added to each well and the plate is covered with transparent tape and placed on the microplate reader. Readings at 414 nm are initiated 45 sec following the addition of ATCHI and are collected at regular intervals for up to 5 min.

Revised April 11, 1990

APPROVE

7. COBAS/FARA Centrifugal Analyzer Assay Procedure:

a. This method was developed as an alternative to the microplate assay procedure for testing the effect of P&T compounds on aging of GD-inhibited AChE. It may be used for studies where the minimum aging time is greater than 1 min. The various incubation times may be changed relative to the demands of the protocol used. The COBAS/FARA is turned on and the GD reactivator program is utilized. This program simulates the microplate assay and contains the following assay parameters:

Measurement Mode: Absorb
Calibration Mode: Factor
Reagent Blank: No Blank

Wavelength: 410 nm
Temperature: 25 C
Decimal Position: 1
Unit: mU/mL

Analysis:

(1) Sample (Enzyme): 10 µL Diluent: 5 µL

(2) Incubation: 5 sec

(3) Reagent 1 (XGD): 10 µL Diluent: 5 µL

(4) Incubation: 5 sec

(5) Reagent 2 (P&T Compound or Assay Buffer): 20 µL
Diluent: 0

(6) Incubation: 5 sec

(7) Reagent 4 (Reactivator; e.g., HI-6): 20 µL

(8) Incubation: 300 sec.

(9) Reagent 3 (DTNB): 150 µL

(10) Incubation: 120 sec

(11) Start Reagent (ACTI): 10 µL

(12) Readings: The total reading time and interval reading time are such to permit accurate kinetic analysis. For monkey erythrocyte cholinesterase, readings are taken at least one reading every 2 min up to a total of 14 min.

APPR

(13) Calculations: The units to which the conversion factor is derived are those mandated by the protocol, otherwise, standardized conversion factors may be calculated as shown below:

Conversion Factor (U/mL) = 0.25/(13.6*0.010) = 1.838
where:

0.25 = square cm area of the cuvette
13.6 = mM absorptivity of DTNB
0.010 = sample volume, in mL, used in absorptivity measurement

Because the values obtained using a factor which results in U/mL are usually less than 1.0, the units may be converted to mU/mL by substitution of 1,838.0 as the conversion factor used in the program. The units may also be expressed as a function of the total reactant volume (i.e., 0.05 mL) by substitution of the total reactant volume value for that of the sample volume in the above equations.

Sample Calculation: The kinetic program on the instrumentation determines change in absorbance per min from the data it collects. This is converted to mU AChE per mL by multiplying by the conversion factor.

AChE (mU/mL) = (change in absorbance/min) * (conversion factor)

b. As required for AChE samples from different species, alterations in the temperature or the volumes of Steps 1 to 5 may be made. If the sample volume is changed, then the conversion factor will need to be changed as indicated in the sample calculation equation. The total volume of Steps 1 to 5 should equal 50 µL. Section 5.a. is repeated at least two times using a different length of incubation with each repetition. The time length used in Step 6 will depend on the species from which the erythrocytes are obtained.

c. This assay may be used for determining the aging rate of erythrocyte AChE by keeping assay buffer as Reagent #2. This may be performed on AChE samples that have not maximally aged by 5 min of GD addition. Section 12.b would be replaced with additional Step 6 times ranging between 5 sec to 16 min. If a longer incubation time than 16 min is required, a second incubation step may be included in between Steps 6 and 7.

Revised April 11, 1990

J. Decontamination: Proper protective equipment and clothing must be utilized throughout these operations in accordance with FSSP SOPs MREF-23 and/or MREF-25. All absorbent material covering the hood surface will be placed in the decontaminating solution after each operation. All disposable glassware in hoods will be submerged in the appropriate decontaminating solution (10 percent NaOH) overnight. All non-disposable glassware in hoods will be) filled with the appropriate decontaminating solution (10 percent NaOH) overnight. Materials left to soak in decontaminating solution overnight will be removed from the hood on the next work day. The glassware, equipment, or non-expendable materials are rinsed with water and removed from the hood. Expendable items may be placed directly into a primary plastic bag within the hood. The primary plastic bag is then sealed with adhesive tape and placed inside another plastic bag, which is then sealed with adhesive tape to provide double containment of decontaminated materials.

Bags of waste must be labeled "Decontaminated Materials" with type of XCSM, date of bagging, bag identification number, and name of person packaging the decontaminated materials in accordance with MREF SOP-83-3, Section H. The double-contained materials can then be incinerated.

K. Emergency Procedures: If a XGD spill occurs, decontamination solution (10 percent NaOH) located within the hood is gently poured or swabbed with soaked absorbent paper held with forceps on the area in an amount that is at least tenfold in excess of the spill. Decontaminating solution is then absorbed with diatomaceous earth or other absorbent and deposited into double plastic bags. The cleaning/absorption procedure is repeated again.

In the event of any incident or exposure, the MREF Manager or his designee must be notified immediately.

L. First Aid Procedures: Make sure that you protect yourself from contamination by the casualty. Mask if in doubt. Personnel exposed to a toxic agent will be removed immediately to a shower area where washing and first aid can be administered by co-workers. If there is any question about the source of contamination, place the victim under the emergency shower. Wash the victim down with soap; do not scrub as this may enhance penetration. Notify MREF on-site medical personnel as quickly as possible.

1. Emergency Treatment for XGD:

a. Emergency decontamination when the source of contact is certain: Remove contaminated clothing. Transfer the victim to clean area and thoroughly decontaminate with 5 percent NaOCl only in the

areas below the eyes in the position in which the victim is being held. Wash skin at once with diluted chlorine-type bleach and rinse with copious amounts of water. Rinse eyes with water only; rinsing a minimum of 10 min at the eyewash fountain. Decontaminate with dry tissue or absorbent paper followed by water in areas close to the eyes. Observe victim for signs and symptoms of GD intoxication.

b. Emergency decontamination when source of contact is uncertain: Place victim in shower and remove clothing. Observe for signs and symptoms of GD intoxication.

c. If victim has symptoms of anticholinesterase poisoning beyond miosis, inject him with the contents of the atropine/2-PAM autoinjectors at intervals of 5-10 min up to a maximum of three injections. Note time of each injection on the victim for reference by physician.

d. If victim has stopped breathing, employ resuscitation with the ambu-bag immediately. Use the atropine autoinjectors after you have successfully succeeded in restoring respiration.

2. At the direction of the MREF Manager or on-site Medical Officer the decontaminated individual may be transported by ambulance to a local hospital for further observation and treatment.

3. In the event of any exposure, the MREF Manager or his designee must be summoned after the immediate emergency is taken care of and informed of the exposure.

M. Data Calculations and Analysis:

1. Using their respective reagent blank values, the GD-inhibited AChE control (EI), reactivator control (ER), and reactivator-treated aged AChE (EIR) values are corrected for any absorbance change contributions from each reactivator on assay reagents. Control AChE (E) values are used as the basis for all percentage estimates of reactivator effectiveness. The values described above are used to calculate the percent reactivation for each reactivator (at each addition time and reactivator concentration tested) using the procedure recommended by Keijer et al.:

$$\% \text{ reactivation} = \frac{EIR \times \frac{E}{ER} - EI}{E - EI} \times 100$$

Revised April 11, 1990

APPROVI

2. These percentage reactivation estimates are then converted to $\log_{10}$ values which are used to compare the ability of each candidate reactivator to inhibit the GD aging rate of AChE at the various addition times versus the similar values for a standard reactivator (which are selected by the protocol used). This comparison is performed using linear regression estimates for the slope where $\log_{10}$ percent reactivation is the dependent variable and time of reactivator addition following GD addition is the dependent variable. A compound that decreases the GD-aging rate as well as or better than the baseline value is considered a positive candidate compound.

M. References:

1. Puu, G., Artursson, E., and Bucht, G., Biochem. Pharmacol., 35(9), 1505, 1986.

2. Keijer, J. H., Wolring, G. Z, and deJong, L. P. A., Biochem. Biophys. Acta, 334,146, 1974.

DWH/tsk

Revised April 11, 1990

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STANDARD OPERATING PROCEDURE
MREF SOP-89-55

TITLE: Analysis and Structural Verification of Atropine in Citrate Buffer

LABORATORY: MREF, HML, or King Ave. SOP APPROVAL DATE: 02/26/90

PLACE OF OPERATION OR TEST: Any safety approved laboratory within the facilities

This Standard Operating Procedure (SOP) has been prepared as prescribed by Contract DAMD17-89-C-9050 and will be effective for one year from date of approval unless sooner rescinded or superseded.

No deviation from this SOP will be permitted. Whenever the approved method is changed, the SOP will be revised.

Supervisory personnel will assure that all personnel involved with this SOP have been properly trained and instructed in its provisions and attest to this requirement by affixing their signatures on page 3.

A copy of this SOP will be posted at the job site whenever the operation is being performed.

Submitted By: Timothy L Hayes 2/20/90
Signature/Date

Timothy L. Hayes, Research Scientist
Printed Name/Title

Approved By: 2/20/90
Signature/Date

Garrett S. Dill, D.V.M., Manager
Printed Name/Title

Approved By: David Stitcher 2/26/90
Signature/Date

David L. Stitcher, CIH, Safety/Surety Officer
Printed Name/Title

Revised February 20, 1990

Approved By:

Richard A. Stewart 2-27-90
Signature/Date

Quality Assurance Unit
Health and Environment Group
Printed Name/Title

Ronald B. Gammie/for 2/27/90
Signature/Date

Charles K. Burdick, Director
Total Quality Program
Health and Environmental Group
Printed Name/Title

Revised February 20, 1990

SIGNATURES

I have read and understand the contents of MREF SOP-89-55.

Signature	Date	Signature	Date
Stephen L. Rivers	3-14-90		
Melissa J. Myers	3/19/90		
Sheri D. Dove	3/30/90		
James A. Bell	4/2/90		
James Cooper	4/2/90		
Raymond L. Cunningham	6/26/90		

Revised February 20, 1990

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STANDARD OPERATING PROCEDURE 89-55

Analysis and Structural Verification of
Atropine Base in Citrate Buffer

A. Statement of Work: This SOP describes the entire procedures for verification of identity and quantitative measurement of atropine free base by high performance liquid chromatography (HPLC). The procedures for structural verification by nuclear magnetic resonance (NMR) of atropine present in drug formulations are also described. The HPLC effort can be conducted at either the MREF, HML or King Avenue, but the NMR requires the facilities at King Avenue.

B. Responsibility:

1. Personnel Qualifications:

All technical staff will be familiar with handling hazardous materials within the laboratory. Personnel performing the following procedures must read and sign this SOP.

2. Leaders: Leaders of each operation will be designated by the Study Director for that operation. Each leader will insure that the following are observed:

a. Only authorized personnel meeting requirements set forth in Section B.1 are allowed in the room during operations.

b. Adequate, approved, protective equipment is available at all times to personnel at their work site.

c. All leader and technical staff responsibilities specified in the MREF or HML FSSP are followed when work is conducted at the respective laboratories.

d. Each MREF and HML employee has been trained in the techniques of administering first aid and self aid.

e. Work under this SOP is performed only in the area(s) or room(s) designated by this SOP.

Revised February 20, 1990

APPROVED

f. No food, beverage, or tobacco product is consumed, used, or brought into the laboratory. The wearing of contact lenses is prohibited in the laboratory.

g. The safety requirements of this SOP, as well as normal laboratory safety, are maintained.

h. All applicable SOPs are read and signed by all technical staff involved in the operation.

3. Technical Staff: Technical staff will be responsible for abiding by requirements set forth in Section B.2. In addition, they must use personal, protective equipment provided and develop safe work habits to protect themselves and fellow workers from injury and to prevent damage to material, equipment, and facilities.

4. Research Organization: The organization involved in this research is the MREF of Battelle Memorial Institute, 505 King Avenue, Columbus, Ohio 43201-2693.

C. Materials To Be Used:

1. Solvents and Chemicals: The atropine sulfate solid which will be used on this program for preparation of analytical standards will be provided by the U.S. Army Medical Research and Development Command (USAMRDC) or a source which can provide an established purity.

If the atropine dosing solution is not received in a pre-packaged form upon receipt, the atropine dosing solution in citrate buffer will be stored in subdued lighting at 4 C. If a pre-packaged form has been received, it will be stored as directed by the supplier.

NMR spectra will be obtained on dilute solutions of the drug dissolved in > 99.8 percent deuterium oxide (Stohler Isotope Chemicals or equivalent). NMR tubes will be the Stohler Isotope Chemicals "Ultra Precision" model or the equivalent model from other manufacturers.

Other materials will include acetonitrile (Burdick and Jackson HPLC Grade), methanol (Burdick and Jackson HPLC Grade), benzene (Burdick and Jackson HPLC Grade), deionized water or millipore water, glacial acetic acid (Baker Reagent Grade), tetrabutylammonium chloride (Aldrich 98+ percent), sodium lauryl sulfate (Aldrich 98 percent), sodium heptane sulfonate (1-heptane sulfonic acid, sodium salt) (Aldrich 98+ percent), tetramethylammonium chloride (Aldrich 98+ percent), and helium or nitrogen gas.

Revised February 20, 1990

APPROVED

D. Equipment: Freezer, refrigerator, labels, first-aid kit, plastic-backed, absorbent paper, squirt bottles, wiping tissues, beakers, bottles, maxi-vials, pipettes, pipette bulbs, tissue paper, laboratory coat, protective eyewear, spatula, stainless-steel pans, glass stir rods, syringes, needles, forceps, and latex gloves.

Proton NMR spectra will be obtained on Battelle's Varian CFT-20 Fourier transform NMR spectrometer located in Room 7237-A of the King Avenue facility.

The HPLC analytical system, to be used consists of the following: HPLC pump, HPLC ultra violet (UV) detector, HPLC injection system (autosampler), analytical column, strip-chart recorder (optional), electronic data system. Any equivalent system may be used once confirmation of performance has been established.

E. Hazards Involved:

1. Solvents: The solvents used in preparing the dilute material may have hazards associated with their use. A copy of the Material Safety Data Sheets (MSDS) is available from the manufacturer or through Battelle's Safety Office at 505 King Avenue. A brief listing of hazards associated with handling the more commonly used solvents has been included:

   a. Acetonitrile: Acetonitrile is a flammable liquid that must be handled as a solvent with a dangerous fire risk. The flash point of acetonitrile is 5.56 C. The 1988-1989 ACGIH TLV for acetonitrile is 40 parts per million (ppm) as an 8-hr TWA and 60 ppm as a 15-min STEL. Skin contact may also represent a significant route of exposure.

   b. Methanol: Methanol is a flammable liquid that must be handled as a solvent with a dangerous fire risk. The flash point (open cup) of methanol is 12.2 C, with an autoignition temperature of 464 C. The 1988-1989 ACGIH TLV for methanol is 200 ppm as an 8-hr TWA and 250 ppm as a 15-min STEL. Also, skin contact may represent a significant route of exposure.

   c. Benzene: Benzene is a flammable liquid that must be handled as a solvent with a dangerous fire risk. Benzene is toxic by ingestion, inhalation, and skin absorption. Benzene is regulated as a carcinogen by the Occupational Safety and Health Administration (OSHA) resulting in excess leukemia. Containers must say "DANGER CONTAINS BENZENE CANCER HAZARD." OSHA 8-hr permissible exposure limit (PEL) = 1 ppm, Action Level = 0.5 ppm.

Revised February 20, 1990

APPROVED

F. Safety Requirements:

1. Hoods: Hood face velocity must average 100 ± 20 lfpm. The average is computed from individual readings taken in approximately each square foot of hood face (usually nine readings). No equipment will be within 20 cm of the face of the hood.

2. Protective Equipment: When working in the laboratory, the following clothing and protective gear are required as a minimum for all personnel. This equipment must be used as directed in the FSSP.

   lab coat
   latex gloves (as needed)
   protective eyewear

   All provisions of the FSSP apply to the checking and testing of gloves, aprons, and other protective equipment.

3. First Aid: The location of the nearest eye-wash fountain, shower, and fire extinguisher will be known to all workers before work begins.

G. Procedures:

1. MREF Entry: Before entering the secured facility, note the status of the "Agent-in-Use" light at the turnstile. If the "Agent-in-Use" lights are turned on, note the room location and be sure that upon entry to the laboratory area all safety equipment and procedures described in FSSP SOP MREF-18 are in place. Upon entry of the room, confirm that there are no audible alarms. No operations can be initiated in a room with audible alarms. After entry, personnel will observe the magnehelic gauge on the hood. If inspection reveals that the hood has failed, is marginal in flow, or operates outside the guidelines of FSSP SOP MREF-21, the problem is reported to the MREF Manager and the operation does not begin.

2. Hood Set Up: The operation hood area must be prepared with all materials necessary to perform the operation prior to starting the operation. All materials will be kept behind the 8-inch line in the hood.

3. Sample Preparation: The drug formulation samples provided for analysis will be manipulated so that the interference of solvents and other components associated with the samples is minimized to provide relatively pure drug samples for NMR analysis.

Revised February 20, 1990

APPROVED

HPLC analyses may be performed on either the dosing formulations as received, dilutions of the parent materials, or on reference standard solutions of known concentration.

a. Analytical Reference Standard: Solid atropine sulfate standard used as a reference material is dried at 100 C, 0.4 mm Hg for 3 hr prior to use in a vacuum oven. This is performed by placing the solid material contained in its original container which has had its cap removed into a pre-heated oven. The oven is sealed and the vacuum adjusted to 0.4 mm Hg.

b. NMR: For the NMR sample preparation, 1 mL of test sample is made basic with 2.0 mL of 0.1 M sodium hydroxide to reach a pH of approximately 13 (verified by color pHast paper). This solution is stirred rapidly with benzene (5.0 mL) for 15 min and then poured through Whatman lps phase separation paper (with 1.0-mL benzene rinse). The filtrate is stirred for 1 min with 2.0-mL deionized water and this mixture is passed again through a fresh phase separation paper (with 1.0-mL benzene rinse). The benzene filtrate is evaporated in a rotary evaporator to yield atropine as its free base. The sulfate is reformed by adding a slight molar excess of dilute D_2SO_4 in D_2O to the free base.

   NMR samples are prepared by transfer of the deuterium oxide solution and transferred into an NMR tube (tube capped after transfer) for NMR analysis.

c. HPLC Analysis: Samples are either analyzed directly or can be diluted so that the expected concentration range is between 0.1 and 1.0 mg/mL.

4. Preparation of Standard Solutions: Standard solutions of atropine sulfate are prepared for NMR reference spectrum and HPLC standard curve determinations.

   a. NMR: Within a glove bag thoroughly flushed with dry nitrogen or argon, weigh 10 ± 0.1 mg of atropine sulfate onto weighing paper. Transfer the sample into a screw-capped bottle and close tightly. Outside the bag, dissolve the sample in an accurately measured volume of 10.0 mL of deuterium oxide and recap the bottle to minimize the contamination of the sample with undeuterated moisture.

   b. HPLC: Weigh 50 ± 0.1 mg of atropine sulfate onto weighing paper. Quantitatively, transfer the sample into a 50-mL volumetric flask containing approximately 40 mL of mobile phase (see Section G.6.b)

APPROVED

Mix the solution thoroughly on a vortex mixer. Dilute to 50.0 mL with the mobile phase and remix the solution. The resulting concentration of the atropine sulfate will be approximately 1 mg/mL.

Mix and dilute the atropine sulfate stock solution with the mobile phase as follows:

10.0-mL stock + 0.0-mL mobile phase
5.0-mL stock + 5.0-mL mobile phase
2.5-mL stock + 7.5-mL mobile phase
1.0-mL stock + 9.0-mL mobile phase
0.0-mL stock + 10.0-mL mobile phase

The atropine sulfate concentrations obtained are 1.00, 0.50, 0.25, 0.10, and 0.0 mg per mL.

Diluted standard solutions are kept refrigerated until use. Standards may be kept refrigerated for up to 30 days.

5. Analysis Start-Up: NMR is performed to verify the structure of atropine sulfate. HPLC is performed to quantitatively determine the concentration of atropine sulfate and confirm the identity of the atropine in the samples.

   a. NMR: Calibrate the NMR instrument and data system according to instructions in the operator's manual. When properly calibrated against the standard reference solutions identified in the manual, proceed with the analysis Section G.7.a.

   b. Quantitative HPLC: Prepare HPLC mobile phase for quantitative analysis by dissolving 2.2 g of sodium heptane sulfonate (1-heptane sulfonic acid sodium salt) and 2.7 g of tetramethylammonium chloride in approximately 90 mL of deionized water. Add 1.0 mL of glacial acetic acid and dilute to 1 L and mix. Filter buffer solution before using.

   The mobile phase may be established using a gradient system with a 78 percent buffer : 2 percent methanol : 20 percent acetonitrile ratio or mixed prior to analysis. To mix the mobile prior to analysis, add 780 mL of the buffer prepared above to a 1-L glass bottle, add 20 mL of methanol and 200 mL of acetonitrile and mix. Once the buffer has been prepared, it must be filtered and used within 30 days.

Revised February 20, 1990

APPROVED

Insure that the appropriate analytical column has been installed in the analytical system, and that the injector is equipped with at least a 20 µL sample injection loop.

All mobile phase must be filtered and degassed for at least 5 min with nitrogen or helium, prior to use.

The detector and the pump must be turned on for a warm-up period of at least 15 min prior to system evaluation. The pump flow must be set at 1.0 mL/min during the warm-up period. After approximately 15 min, measure the flow for 5 min with a 10-mL graduated cylinder. The flow rate must be set at 1.0 ± 0.1 mL/min. Adjust the flow rate setting on the pump controller if necessary to obtain an actual flow rate within these limits and re-check flow.

After the pump has been on for 30 min, adjust the detector zero with the balance control with the detector atteruation set at the appropriate attenuation. Adjust the recorder to electrical zero at "0" chart units. Adjust the detector zero to slightly above the electrical zero position with the recorder balance control.

c. HPLC Identity Confirmation: Prepare HPLC mobile phase for identity confirmation by adding 6.0 g of sodium lauryl sulfate and 1.0 g of tetrabutylammonium nitrate to a 1-L volumetric flask and dissolve the reagents in approximately 500 ml of deionized water. Add 20 mL of glacial acetic acid to the solution and mix. The volumetric flask is filled to the 1-L mark and the solution re-mixed. Filter the solution with a 5 µm filter and store in a clean glass bottle. Use within 30 days.

The mobile phase may be established using a gradient system with a 60 percent buffer : 40 percent acetonitrile ratio or mixed prior to analysis. To mix the mobile prior to analysis, add 600 mL of the buffer prepared above to a 1-L glass bottle and add 400 mL of acetonitrile and mix. Once the buffer has been prepared it must be used within 30 days.

Insure that a Supelco LC-1 column or equivalent has been connected to the injector and detector and the injector is equipped with a 20 µL sample injection loop.

All mobile phase must be degassed for at least 5 min with helium or nitrogen prior to use.

Revised February 20, 1990

The detector and the pump must be turned on for a warm-up period of at least 15 min prior to system evaluation. The pump flow must be set at 1.0 mL/min during the warm-up period. After approximately 15 min, measure the flow for 5 min with a 10-mL graduated cylinder. The flow rate should be 1.0 ± 0.1 mL/min. Adjust the flow rate setting on the pump if necessary to obtain an actual flow rate within these limits and re-check.

After the pump has been on for 30 min, adjust the detector zero with the balance control with the detector set at the appropriate attenuation. Adjust the recorder to electrical zero at "0" chart units. Adjust the detector zero to slightly above the electrical zero position with the recorder balance control.

6. Analysis of Samples: NMR is performed for structural confirmation. HPLC standards and collected samples are analyzed to determine concentration and identity confirmation.

   a. NMR: Multiple acquisitions (> 100 transients) are generally required. Spectra will be printed on standard NMR paper and computer referenced to the chemical shift of sodium 2,2-dimethyl-2-silapentane-5-sulfonate determined on the same day to facilitate interpretation.

   b. Quantitative HPLC: The following is a set of HPLC conditions that have been found to be satisfactory for quantitative analysis of atropine sulfate by HPLC (reference 1):

      Column: C18 u-Bondapak or equivalent, 250-mm long x 4.6-mm inner diameter with 5 micron particle size.

      Mobile Phase: See Section 6.6.b

      Detector: UV @ 260 nm

      Flow Rate: 1.8 mL/min

      Injection Volume: 20 µL

      For quantitative analysis of atropine sulfate samples, transfer 1-mL duplicate aliquots of each atropine sulfate standard to autosampler vials and place the vials in the autosampler in ascending concentration order. Set up the data system to acquire data for each standard as described in the instruction manual. Transfer 1-mL duplicate aliquots of each sample to autosampler vials and place the vials in the autosampler.

Revised February 20, 1990

For every ten samples to be analyzed, one blank sample and one standard must be analyzed as a minimum. All samples must be analyzed under the same conditions as used for the standards.

c. HPLC Identity Confirmation: For confirmation of the identity of atropine sulfate by HPLC, a second set of HPLC conditions is employed. The following is a set of HPLC conditions found to be satisfactory for the confirmation of atropine.

Column: Supelco LC-1, 250-mm long x 4.6-mm inner diameter, with 5 micron particle size.

Mobile Phase: See Section G.6.c

Detector: UV @ 254 nm

Flow Rate: 1 mL/min

Injection Volume: 20 µL

For confirmation purposes, analyze an atropine sulfate standard and a sample from the formulation under these HPLC conditions.

7. Instrument Shut-Down:

a. When the instrument is not to be used for extended periods of time, the system must be shut down following manufacturer's instructions to ensure column life and instrument stability.

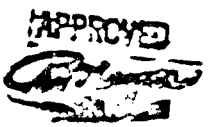
b. For overnight shut-down, turn off the UV detector, chart recorder, and pump controller.

c. For weekend shut-down, follow the same procedure as for overnight shut-down but also cap off the analytical column to prevent the solid phase from drying.

8. Data Reduction: The NMR spectra obtained in Section G.7 are compared to reference NMR spectra for atropine to verify structural identity. The HPLC samples analyzed in Section G.7 are compared with results obtained from known reference standards to determine concentration.

a. NMR: Compare the NMR spectrum for the sample with the spectrum obtained for the atropine sulfate reference standard. Verify correspondence of chemical shifts, multiplicities, and intensities for structural verification in conjunction with HPLC findings.

Revised February 20, 1990



b. Quantitative HPLC: Obtain printouts of the peak areas for each standard and sample as described in the data system instruction manual. Prepare a standard curve from the peak areas versus concentration of the standards

Determine the atropine sulfate concentration in the samples and control standards using the standard curve. If necessary, correct any dilution made to the samples prior to analysis.

If the response for any of the control standards varies from the predicted response by more than ± 10 percent, then the samples associated with that standard are reanalyzed.

c. HPLC Identity Confirmation: HPLC confirmation of the identity of atropine sulfate is performed by analysis under a second set of HPLC conditions. Compare the retention times and relative responses of the atropine sulfate reference standard and sample peak for structural confirmation in conjunction with the first set of HPLC results and NMR conclusions.

H. Emergency Procedures: All personnel involved in the HML or MREF laboratory operations, must be familiar with the respective laboratory's FSSP, and the emergency procedures detailed within this document. All personnel involved in the King Avenue operation must be familiar with HEG H/SP E-01 and the emergency procedures detailed within this document.

I. First Aid Procedures: First aid and self aid at the MREF are to be conducted as specified in the FSSP.

J. References:

1. "Assay of Formulated Atropine Solution, WR-6241AK, B107753, Lot No. RU7144," Report No. 527, Contract No. DAMD17-85-C-5141, SRI International Project No. 8504, December 10, 1985.

TLH:cah

Revised February 20, 1990

STANDARD OPERATING PROCEDURE
MREF SOP-89-62

TITLE: Extraction and Analysis of α 1-(2-Hydroxyiminomethylpyridinium)-2-(4-Carboxyamido-Pyridinium)-Dimethyl Ether Dichloride (HI-6) from Blood Using High Performance Liquid Chromatography (HPLC)

LABORATORY: MREF, HML, or King Ave. SOP APPROVAL DATE: June 18, 1990

PLACE OF OPERATION OR TEST: Any safety approved laboratory within the approved facilities

This Standard Operating Procedure (SOP) has been prepared as prescribed by Contract DAMD17-89-C-9050 and will be effective for one year from date of approval unless sooner rescinded or superseded.

No deviation from this SOP will be permitted. Whenever the approved method is changed, the SOP will be revised.

Supervisory personnel will assure that all personnel involved with this SOP have been properly trained and instructed in its provisions and attest to this requirement by affixing their signatures on page 3.

A copy of this SOP will be posted at the job site whenever the operation is being performed.

Revised By: Timothy L Hayes 4/3/90
Signature/Date

Timothy L. Hayes, Principal Research Scientist
Printed Name/Title

Approved By: 6/11/90
Signature/Date

Garrett S. Dill, D.V.M., Manager
Printed Name/Title

Approved By: David Stitcher 6/18/90
Signature/Date

David L. Stitcher, CIH, Safety/Surety Officer
Printed Name/Title

APPROVED

Revised June 7, 1990

Approved By:

Richard A. Fiala 6-19-90
Signature/Date

Quality Assurance Unit
Health and Environment Group
Printed Name/Title

Charles K. Burdick 6/21/90
Signature/Date

Charles K. Burdick, Director
Total Quality Program
Health and Environment Group
Printed Name/Title

APPROVED

Revised June 7, 1990

SIGNATURES

I have read and understand the contents of MREF SOP-89-62.

Signature	Date	Signature	Date
[signature]	6-25-90		
[signature]	6-25-90		

Revised June 7, 1990

STANDARD OPERATING PROCEDURE 89-62

Extraction and Analysis of α 1-(2-Hydroxyiminomethylpyridinium)-2-(4-Carboxyamido-Pyridinium)-Dimethyl Ether Dichloride (HI-6) from Blood Using High Performance Liquid Chromatography (HPLC)

A. Statement of Work: This SOP describes the method for the determination of α 1-(2-hydroxyiminomethylpyridinium)-2-(4-carboxyamido-pyridinium)-dimethyl ether dichloride (HI-6) in whole blood. The method incorporates centrifugation, acidification, and liquid-liquid extraction for sample clean-up. The prepared sample is analyzed by high performance liquid chromatography (HPLC). The sample preparation and analysis methods detailed here were developed in support of pharmacokinetics studies performed at Battelle's Medical Research and Evaluation Facility (MREF).

B. Responsibility:

All technical staff will be familiar with the safe handling practices of chemical materials within a laboratory. Personnel performing the following procedures must read and sign this SOP. They must use personal, protective equipment required by the Facility Safety and Surety Plan (FSSP) while working within the MREF and develop safe work habits to protect themselves and fellow workers from injury and to prevent damage to material, equipment, and facilities.

The organization involved in this research is the MREF of Battelle Columbus Operations, 505 King Avenue, Columbus, OH 43201-2693.

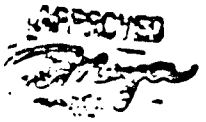
C. Materials To Be Used: Potassium phosphate dibasic, 1-heptanesulfonic acid, triethylamine, acetonitrile, chloroform, isopropyl alcohol, methyl alcohol, trichloroacetic acid, HI-6, 1,1'-methylene-bis [4-(hydroxyiminomethyl) pyridinium] dichloride (MMB-4), acetic acid, and potassium hydroxide, labels, plastic-backed absorbent paper, brown paper, and wiping tissues.

D. Equipment: Freezer, refrigerator, first aid kit, plastic-backed, squirt bottles, beakers, bottles, maxi-vials, pipettes, pipette bulbs, laboratory coat, safety glasses, spatula, stainless-steel pans, glass stir rods, syringes, needles, forceps, and latex gloves.

E. Hazards Involved:

1. Solvents and Chemicals: The solvents and chemicals used in this SOP may have hazards associated with their use. The Material Safety Data Sheets (MSDS) are available in the MREF office files or through Battelle's Safety Services Department at 505 King Avenue.

Revised June 7, 1990



F. Procedures:

1. MREF Entry: Before entering the secured facility, note the status of the "Agent-in-Use" light at the turnstile. If the "Agent-in-Use" lights are turned on, note the room location and be sure that upon entry to the laboratory area that all safety equipment and procedures described in FSSP SOP MREF-18 are in place. Upon entry of the room, confirm that there are no audible alarms. No operations can be initiated in a room with audible alarms. After entry, personnel will observe the magnehelic gauge on the hood. If inspection reveals that the hood has failed, is marginal in flow, or operates outside the guidelines of FSSP SOP MREF-21, the problem is reported to the MREF Manager and the operation does not begin.

2. Hood Set Up: The operation hood area must be prepared with all materials necessary to perform the operation prior to starting the operation. All materials will be kept behind the 8-inch line in the hood.

   Plastic-backed, absorbent paper must be used to protect the work surface of the hood.

3. Solution Preparation:

   a. Mobile Phase Buffer: Accurately weigh 4.1 ± 0.01 g 1-heptanesulfonic acid, and 17.4 ± 0.01 g potassium phosphate dibasic onto weighing paper. Quantitatively transfer each chemical into a 2-L volumetric flask containing approximately 1500 mL millipore water. Deliver 2.81 mL ± 0.01 of triethylamine (using a 5,000-µL gas-tight syringe) to the resulting solution and mix well. Adjust the pH of the solution to 4.1 ± 0.1 with glacial acetic acid (approximately 28 mL). Mix well and dilute to volume with millipore water. Filter through a 0.45 µm filter.

   b. 20.0 mg/mL MMB-4 Stock Solution: Weigh 100 ± 0.01 mg of neat MMB-4 in a weighing dish. Quantitatively transfer the weighed material into a 5-mL volumetric flask containing approximately 2-mL millipore water. Vortex mix the solution for approximately 30 seconds and dilute to volume with millipore water. Vortex mix the final solution for an additional 30 seconds and transfer to a teflon cap lined screw cap vial for storage and label appropriately.

   c. Mobile Phase Diluent with Internal Standard: Accurately measure and dispense 625 µL MMB-4 stock solution at 20.0 mg/mL (using a 1,000 µL gas-tight syringe) into a 100-mL volumetric flask containing approximately 50 mL mobile phase buffer. Mix well. Dilute to volume with mobile phase buffer. Mix resulting solution again, label and store in refrigerator until use.

Revised June 7, 1990

APPROVED

Each day before using diluent, remove a 15 mL aliquot and pH solution to pH 6.2 with a solution of concentrated potassium hydroxide (KOH) (< 0.05 mL).

Prepare the concentrated KOH solution by dispensing approximately 1 g of KOH into a 50-mL beaker and add enough millipore water to dissolve the KOH. CAUTION: Process is extremely exothermic.

d. HI-6 Stock and Spiking Solutions: Two standard solutions of HI-6 are prepared for the purpose of spiking whole blood. These spiked whole blood samples are used as analytical standards.

(1) 10.1 mg/mL HI-6 Stock Solution: Accurately weigh 100 ± 1 mg of HI-6 on weighing paper. Quantitatively transfer the HI-6 into a 10-mL volumetric flask containing approximately 5 mL millipore water. Mix well until dissolved. Dilute to volume with millipore water and mix again. Store the solution in a teflon lined screw cap vial and label appropriately.

(2) 0.506 mg/mL HI-6 Spiking Solution: Into a 10-mL volumetric flask containing approximately 5 mL millipore water, deliver 500 µL of the 10.1 mg/mL HI-6 stock prepared in Section F.3.d.(1) (using a 500-µL gas-tight syringe). Mix well and dilute to volume with millipore water. Mix on a vortex mixer. Store the solution in a teflon lined screw cap vial and label appropriately.

e. Preparation of Acetonitrile-Isopropyl Alcohol (ACN-IPA) Solution: Pour 200 ± 1 mL of spectroscopic grade IPA into a 1-L volumetric flask. Fill the remainder of the volume with ACN and mix thoroughly. Store the solution in a teflon lined screw-cap vial and label appropriately.

f. Preparation of HI-6 Analytical Standards and Blank:

(1) 101-µg/mL Analytical Standard: Into a 13 x 100-mm round bottom polypropylene test tube containing 10 ± 0.1 mL of whole blood, add 100 ± 1 µL of the 10.1 mg/mL HI-6 stock solution prepared in Section F.3.d.(1) using a 100-µL gas-tight syringe. Cap and gently mix the solution by rotating the test tube end over end for 10 rotations. Place the capped test tube into a refrigerated centrifuge at 25 C and centrifuge for 15 min at 1,500 x g. After the solution has separated use a micropipette to transfer the top "clear" plasma layer to another 13 x 100-mm test tube for extraction. Be careful to label the tube with the spiked concentration and date. The samples are stored in the freezer at -20 C until used.

APPROVED

(2) 50.6-µg/mL Analytical Standard: Into a 13 x 100-mm round bottom polypropylene test tube containing 10 ± 0.1 mL of whole blood, add 50 ± 0.1 µL of the 10.1 mg/mL HI-6 stock solution prepared in Section F.3.d.(1) using a 50-µL gas-tight syringe. Cap and gently mix the solution by rotating the test tube end over end for 10 rotations. Place the capped test tube into a refrigerated centrifuge at 25 C and centrifuge for 15 min at 1,500 x g. After the solution has separated use a micropipette to transfer the top "clear" layer to another 13 x 100-mm test tube for extraction. Be careful to label the tube with the spiked concentration and date. The samples are stored in the freezer at -20 C until used.

(3) 25.3-µg/mL Analytical Standard: Into a 13 x 100-mm round bottom polypropylene test tube containing 10 ± 0.1 mL of whole blood, add 25 ± 0.1 µL of the 10.1 mg/mL HI-6 stock solution prepared in Section F.3.d.(1) using a 25-µL gas-tight syringe. Cap and gently mix the solution by rotating the test tube end over end for 10 rotations. Place the capped test tube into a refrigerated centrifuge at 25 C and centrifuge for 15 min at 1,500 x g. After the solution has separated use a micropipette to transfer the top "clear" layer to another 13 x 100-mm test tube for extraction. Be careful to label the tube with the spiked concentration and date. The samples are stored in the freezer at -20 C until used.

(4) 10.1-µg/mL Analytical Standard: Into a 13 x 100-mm round bottom polypropylene test tube containing 10 ± 0.1 mL of whole blood, add 10 ± 0.1 µL of the 10.1 mg/mL HI-6 stock solution prepared in Section F.3.d.(1) using a 10-µL gas-tight syringe. Cap and gently mix the solution by rotating the test tube end over end for 10 rotations. Place the capped test tube into a refrigerated centrifuge at 25 C and centrifuge for 15 min at 1,500 x g. After the solution has separated use a micropipette to transfer the top "clear" layer to another 13 x 100-mm test tube for extraction. Be careful to label the tube with the spiked concentration and date. The samples are stored in the freezer at -20 C until used.

(5) 5.06-µg/mL Analytical Standard: Into a 13 x 100-mm round bottom polypropylene test tube containing 10 ± 0.1 mL of whole blood, add 100 ± 1 µL of the 0.506 mg/mL HI-6 stock solution prepared in Section F.3.d.(2) using a 100-µL gas-tight syringe. Cap and gently mix the solution by rotating the test tube end over end for 10 rotations. Place the capped test tube into a refrigerated centrifuge at 25 C and centrifuge for 15 min at 1,500 x g. After the solution has

APPROVED

separated use a micropipette to transfer the top "clear" layer to another 13 x 100-mm test tube for extraction. Be careful to label the tube with the spiked concentration and date. The samples are stored in the freezer at -20 C until used.

(6) 2.53-µg/mL Analytical Standard: Into a 13 x 100-mm round bottom polypropylene test tube containing 10 ± 0.1 mL of whole blood, add 50 ± 0.1 µL of the 0.506 mg/mL HI-6 stock solution prepared in Section F.3.d.(2) using a 50-µL gas-tight syringe. Cap and gently mix the solution by rotating the test tube end over end for 10 rotations. Place the capped test tube into a refrigerated centrifuge at 25 C and centrifuge for 15 min at 1,500 x g. After the solution has separated use a micropipette to transfer the top "clear" layer to another 13 x 100-mm test tube for extraction. Be careful to label the tube with the spiked concentration and date. The samples are stored in the freezer at -20 C until used.

(7) 1.01-µg/mL Analytical Standard: Into a 13 x 100-mm round bottom polypropylene test tube containing 10 ± 0.1 mL of whole blood, add 20 ± 0.1 µL of the 0.506 mg/mL HI-6 stock solution prepared in Section F.3.d.(2) using a 25-µL gas-tight syringe. Cap and gently mix the solution by rotating the test tube end over end for 10 rotations. Place the capped test tube into a refrigerated centrifuge at 25 C and centrifuge for 15 min at 1,500 x g. After the solution has separated use a micropipette to transfer the top "clear" layer to another 13 x 100-mm test tube for extraction. Be careful to label the tube with the spiked concentration and date. The samples are stored in the freezer at -20 C until used.

(8) 0.506-µg/mL Analytical Standard: Into a 13 x 100-mm round bottom polypropylene test tube containing 10 ± 0.1 mL of whole blood, add 10 ± 0.1 µL of the 0.506 mg/mL HI-6 stock solution prepared in Section F.3.d.(2) using a 10-µL gas-tight syringe. Cap and gently mix the solution by rotating the test tube end over end for 10 rotations. Place the capped test tube into a refrigerated centrifuge at 25 C and centrifuge for 15 min at 1,500 x g. After the solution has separated use a micropipette to transfer the top "clear" layer to another 13 x 100-mm test tube for extraction. Be careful to label the tube with the spiked concentration and date. The samples are stored in the freezer at -20 C until used.

Revised June 7, 1990

APPROVED

(9) 0.0-µg/mL Analytical Blank: Into a 13 x 100-mm round bottom polypropylene test tube containing 10 ± 0.1 mL of whole blood, add 100 ± 1 µL of the millipore water used in preparation of spiking solution using a 100 µL syringe. Cap and gently mix the solution by rotating the test tube end over end for 10 rotations. Place the capped test tube into a refrigerated centrifuge at 25 C and centrifuge for 15 min at 1,500 x g. After the solution has separated use a micropipette to transfer the top "clear" layer to another 13 x 100-mm test tube for extraction. Be careful to label the tube with the spiked concentration and date. The samples are stored in the freezer at -20 C until used.

4. Equipment Preparation:

a. Instrument Preparation: The HPLC is prepared for use with the following recommended initial settings:

(1) Column - 15 cm x 4.6 mm inside diameter (I.D.) Zorbax LC-8 with 5 µm partial size.

(2) Guard Column - 2 cm x 4.6 mm I.D.
Zorbax LC-8 with 5 µm partial size.

(3) Mobile Phase: 80 percent buffer (see Section F.3.a)
5 percent ACN-IPA mixture (see Section F.3.e)
15 percent spectroscopic grade methyl alcohol.

(4) Mobile Phase Flow Rate: 1.5 mL/min.

(5) Injection Loop: 20 µL volume.

(6) Detector Wavelength: 304 nm.

(7) Absorbance Units Full Scale (A.U.F.S.): 0.02.

b. Column Conditioning: The column needs approximately 45 min of mobile phase conditioning before it can be used to analyze samples. This conditioning insures that all stationary phase has reacted with the ion-pair reagent.

c. Column Check: The integrity of the column needs to be checked before samples are analyzed. The proteins and impurities in the blood samples can adhere to the guard column's stationary phase and frits causing a reduction in resolution. To remedy this problem the column must be cleaned and the guard column changed. To determine when these maintenance procedures need to be done a 25 µg/mL HI-6 standard can be analyzed and the resolution between the HI-6 and MMB-4 is determined. When reduction in resolution is observed, the column must either be cleaned or replaced. The

APPROVED

guard column should always be replaced and conditioned without the analytical column in place.

5. Spiking and Extraction Procedures: The calibration standards and unknown samples are prepared using the same procedures detailed below.

   a. Using a 1,000-µL pipettor, place 1,000 µL of either a plasma unknown, or calibration sample into a prelabeled 1.5-mL microcentrifuge tube.

   b. Spike each microcentrifuge tube with 30 µL of a 50 percent trichloracetic acid solution prepared in water. Vortex mix the solution until a milky suspension is observed. Centrifuge the solution for 10 min at 10,000 x g to remove the suspended material from solution. Remove the clear supernatant layer using a disposable transfer pipet and dispense into prelabeled 13 x 100-mm test tube. The lower precipitate layer can be discarded at this time.

   c. Spike each test tube prepared in Section F.6.b with 250 µL of mobile phase diluent prepared in Section F.3.c using a 250-µL gas-tight syringe and mix the solution thoroughly using a vortex mixer.

   d. Into each test tube prepared in Section F.6.c, dispense 5 ± 0.05 mL of chloroform using a calibrated autopipettor.

   e. Cap each tube securely and place into a test tube rack. Place the rack of samples onto a rotating mixer and rotate the samples for approximately 15 min at 30 revolutions per min (rpm). The solutions will turn extremely milky in color indicating the precipitation of the protein materials within the plasma matrix. Once this initial precipitation of the protein has been accomplished, reduce the rotation speed to 10 rpm and continue to operate for an additional 45 min. This provides a 1 hr extraction period.

   f. The rack containing the tubes is removed from the rotating extractor and the resulting solutions centrifuged for 1 hr at 1,500 x g. This step should remove all solid material from the supernatant layer. Be sure that the centrifuge brake has been adjusted so that the solid layer is not disrupted when the centrifuge is stopping.

   g. The clear aqueous supernatant layer is removed from each sample and dispensed into labeled autoanalyzer vials. The sample vials are tightly capped and made ready for analysis.

APPROVED

6. Instrument Calibration: Instrument calibration must be performed each time quantitation of samples is required. The instrument calibration is performed by injecting 20 ± 0.1 µL each of the extracted plasma blank and the analytical standards prepared in Section F.3.f using an autosampler. A complete set of analytical standards is analyzed prior to analysis of any sample extracts. Once calibration of the instrument has been performed and the linearity checked, the sample extracts are analyzed with at least every sixth sample being an analytical standard to check the calibration of the instrument. A complete set of analytical standards is analyzed following the last sample. All analytical standards analyzed are used to develop a complete calibration curve for quantitation of the sample extracts. No sample amount may be reported that exceeds the range of the analytical standards. Samples that yield responses less than the calibration range will be reported as less than the lower quantitation limit. Any sample response that exceeds the largest analytical standard will be reported as greater than the highest analytical standard, and must be either diluted to within range or the calibration range extended for quantification of the sample.

7. Analysis of Analytical Blank: The analytical blank prepared in Section F.3.f.9. is analyzed to determine the quality of the reagents used, and to determine if the presence of sample matrix components interfere with the analysis method.

8. Analysis of Samples: Samples and calibration standards are analyzed using the sample procedures. At least every sixth analysis should be a analytical standard or analytical blank.

9. Calculations: The sample amounts are calculated using an internal standard method of calibration. The calibration data is analyzed using a linear regression model to estimate the parameter values for the model.

   a. Using a simple linear regression program, enter the peak area ratio of HI-6 to MMB-4 as the ordinate (y-value) and the corresponding standard concentration as the abscissa (x-value). The regression model used to generate the slope, intercept, and correlation coefficient for HI-6 in the calibration data is:

$$y = bx + a$$

   b. If a regression program is not available, program the following calculations:

$$b = \frac{[(\Sigma y)(\Sigma x^2)-(\Sigma x)(\Sigma xy)]}{[n(\Sigma x^2)-(\Sigma x)^2]}$$

$$a = \frac{[n(\Sigma xy)-(\Sigma x)(\Sigma y)]}{[n(\Sigma x^2)-(\Sigma x)^2]}$$

$$r = \frac{[n(\Sigma xy)-(\Sigma x)(\Sigma y)]}{[(n(\Sigma x^2)-(\Sigma x)^2)^{\frac{1}{2}}(n\Sigma(y^2)-(\Sigma y)^2)^{\frac{1}{2}}]}$$

where,

y = ax + b
a = slope
b = y-intercept
r = correlation coefficient
x = peak area
y = amount GD in ng
n = number of replicates

c. All data points obtained from the analysis of the analytical standards are used to calculate the regression model estimates. In addition the percent relative standard deviation (%RSD) between replicate standards should be monitored to insure that the instrument response does not change excessively during analysis. The %RSD for any set of analytical standards should not be greater than 10 percent. If the %RSD for any set is greater than 10 percent, the analysis is stopped and the problem corrected prior to resuming analyses. Do not include the results of the analytical blank analyses in the calibration calculations as this will weigh the regression toward zero.

d. Identify the analyte and internal standard peaks in the unknown sample chromatograms and record each peak area. The ratio of the analyte versus the internal standard is calculated and the value recorded. Using the regression values calculated from the calibration data, calculate the concentration for each sample using the formula above.

10. Column Clean-Up: After each set of samples are analyzed, the column must be cleaned to remove proteins and peptides adhering to the stationary phase. This is accomplished by flushing the column with a series of solvents over a period of time. The sequence recommended for this clean-up is:

Millipore water at 1.5 mL/min for 60 min.

ACN at 1.5 mL/min for 10 min.

IPA at 1.0 mL/min 10 min.

Revised June 7, 1990

APPROVED

50:50 IPA/chloroform ramped program
1.5 mL/min - 2.0 mL/min in 10 min
- 2.0 mL/min for 50 min

IPA at 1.5 mL/min for 5 min.
ACN at 1.5 mL/min for 10 min.

11. Instrument Shut-Down: When the instrument is not to be used for extended periods of time, the system must be shut down following manufacturer's instructions to ensure column life and instrument stability. The column clean-up procedure (Section G.10) is followed and the column is stored with 100 percent ACN wetting the stationary phase. The column must be tightly capped during storage to prevent solvent evaporation. The column must never be stored with buffer remaining on the support.

TLH/tsk

Revised June 7, 1990

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STANDARD OPERATING PROCEDURE
MREF SOP-89-64

TITLE: Analysis of α 1-(2-Hydroxyiminomethylpyridinium)-2- (4-Carboxyamido-Pyridinium)-Dimethyl Ether Dichloride (HI-6) and 1,1'Methylene-Bis [4(Hydroxyiminomethyl)Pyridinium] Dichloride (MMB-4) Using High Performance Liquid Chromatography (HPLC)

LABORATORY: MREF,HML, or King Ave. SOP APPROVAL DATE: June 16, 1990

PLACE OF OPERATION OR TEST: Any safety approved laboratory within the approved facilities

This Standard Operating Procedure (SOP) has been prepared as prescribed by Contract DAMD17-89-C-9050 and will be effective for one year from date of approval unless sooner rescinded or superseded.

No deviation from this SOP will be permitted. Whenever the approved method is changed, the SOP will be revised.

Supervisory personnel will assure that all personnel involved with this SOP have been properly trained and instructed in its provisions and attest to this requirement by affixing their signatures on page 3.

A copy of this SOP will be posted at the job site whenever the operation is being performed.

Submitted By: Timothy L Hayes 6/7/90
Signature/Date

Timothy L. Hayes, Principal Research Scientist
Printed Name/Title

Approved By: 6/11/90
Signature/Date

Garrett S. Dill, D.V.M., Manager
Printed Name/Title

Approved By: 6/18/90
Signature/Date

David L. Stitcher, CIH, Safety/Surety Officer
Printed Name/Title

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Approved By:

Richard A. Swanb 6-19-90
Signature/Date

Quality Assurance Unit
Health and Environment Group
Printed Name/Title

Chas K. Burdick 6/21/90
Signature/Date

Charles K. Burdick, Director
Total Quality Program
Health and Environment Group
Printed Name/Title

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SIGNATURES

I have read and understand the contents of MREF SOP-89-64.

Signature	Date	Signature	Date
Sheri D. Moore	6/20/90		
Timothy K. Hager	6/20/90		

STANDARD OPERATING PROCEDURE 89-64

Extraction and Analysis of α 1-(2-Hydroxyiminomethylpyridinium)-2-(4-Carboxyamido-Pyridinium)-Dimethyl Ether Dichloride (HI-6) and 1,1'-Methylene-Bis [4-(Hydroxyiminomethyl) Pyridinium] Dichloride (MMB-4) Using High Performance Liquid Chromatography (HPLC)

A. Statement of Work: This SOP describes the method for the determination of α 1-(2-hydroxyiminomethylpyridinium)-2-(4-carboxyamido-pyridinium)-dimethyl ether dichloride (HI-6) and 1,1'-methylene-bis [4-(hydroxyiminomethyl) pyridinium] dichloride (MMB-4) in dosing samples. The prepared sample is analyzed by high performance liquid chromatography (HPLC). The sample preparation and analysis methods detailed here were developed in support of pharmacokinetics studies performed at Battelle's Medical Research and Evaluation Facility (MREF). This method uses HI-6 as the internal standard when analyzing for MMB-4 and MMB-4 as the internal standard when analyzing for HI-6. Only samples containing one of the analytes can be analyzed using this SOP.

B. Responsibility:

All technical staff will be familiar with the safe handling practices of chemical materials within a laboratory. Personnel performing the following procedures must read and sign this SOP. They must use personal, protective equipment required by the Facility Safety and Surety Plan (FSSP) while working within the MREF and develop safe work habits to protect themselves and fellow workers from injury and to prevent damage to material, equipment, and facilities.

The organization involved in this research is the MREF of Battelle Memorial Institute, 505 King Avenue, Columbus, OH 43201-2693.

C. Materials To Be Used: Potassium phosphate dibasic, 1-heptanesulfonic acid, triethylamine, acetonitrile, methyl alcohol, trichloroacetic acid, HI-6, 1,1'-methylene-bis [4-(hydroxyiminomethyl) pyridinium] dichloride (MMB-4), acetic acid, labels, plastic-backed absorbent paper, brown paper, and wiping tissues.

D. Equipment: Freezer, refrigerator, first aid kit, plastic-backed, squirt bottles, beakers, bottles, maxi-vials, pipettes, pipette bulbs, laboratory coat, protective eyewear, spatula, stainless-steel pans, glass stir rods, syringes, needles, forceps, and latex gloves.

E. Hazards Involved:

1. Solvents and Chemicals: The solvents and chemicals used in this SOP may have hazards associated with their use. The Material Safety Data

Sheets (MSDS) are available in the administrative area of the MREF or through Battelle's Safety Services Department at 505 King Avenue.

F. Procedures:

1. Hood Set Up: The operation hood area must be prepared with all materials necessary to perform the operation prior to starting the operation. All materials will be kept behind the 8-inch line in the hood.

   Plastic-backed, absorbent paper must be used to protect the work surface of the hood.

2. Solution Preparation:

   a. Mobile Phase Buffer: Accurately weigh 4.1 ± 0.01 g 1-heptanesulfonic acid, and 17.4 ± 0.01 g potassium phosphate dibasic onto weighing paper. Quantitatively transfer each chemical into a 2-L volumetric flask containing approximately 1500 mL millipore water. Deliver 2.81 mL ± 0.01 of triethylamine (using a 5000-µL gas-tight syringe) to the resulting solution and mix well. Adjust the pH of the solution to 4.1 ± 0.1 with glacial acetic acid (approximately 28 mL). Mix well and dilute to volume with millipore water. Filter through a 0.45 µm filter.

   b. 20.0 mg/mL MMB-4 Stock Solution: Weigh 100 ± 1.0 mg of neat MMB-4 in a weighing dish. Quantitatively transfer the weighed material into a 5-mL volumetric flask containing approximately 2-mL millipore water. Vortex mix the solution and dilute to volume with millipore water. Stock solution must be made up fresh daily.

   c. Mobile Phase Diluent with MMB-4 Internal Standard: Accurately measure and dispense 125 µL MMB-4 stock solution at 20.0 mg/mL (using a 250 µL gas-tight syringe) into a 100-mL volumetric flask containing approximately 50 mL mobile phase buffer. Mix well. Dilute to volume with mobile phase buffer. Mix resulting solution again, label and store in refrigerator until use.

   d. 10.0 mg/mL HI-6 Stock Solution: Accurately weigh 100.0 mg ± 1.0 mg of HI-6 on weighing paper. Quantitatively transfer the HI-6 into a 10 mL volumetric flask containing approximately 5 mL millipore water. Mix well until dissolved. Dilute to volume with millipore water and mix again. Stock solution must be made up fresh daily.

   e. Mobile Phase Diluent with HI-6 Internal Standard: Accurately measure and dispense 1,110 µL of the HI-6 stock solution at

10.0 mg/mL (using a 1,250 µL gas-tight syringe) into a 100-mL volumetric flask containing approximately 50 mL mobile phase buffer. Mix well. Dilute to volume with mobile phase buffer. Mix resulting solution again, label and store in refrigerator until use.

f. HI-6 Standards: (Must be made up fresh daily.)

(1) 100 µg/mL HI-6 Standard: Into a 10 mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with MMB-4 (G.3.c.), deliver 100 µl of the 10 mg/mL HI-6 stock solution (using a 100 8 l gas-tight syringe). Mix well and dilute to volume with mobile phase diluent.

(2) 50 µg/mL HI-6 Standard: Into a 10-mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with MMB-4, deliver 50 µL of the 10 mg/mL HI-6 stock prepared in Section G.3.d. (using a 50 µL gas-tight syringe). Mix well and dilute to volume with mobile phase diluent. Mix on a vortex mixer.

(3) 25 µg/mL HI-6 Standard: Into a 10 mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with MMB-4, deliver 25 µL of the 10 mg/mL HI-6 stock prepared in Section G 3.d. (using a 25 µL gas-tight syringe). Mix well and dilute to volume with mobile phase diluent. Mix on a vortex mixer.

(4) 10 µg/mL HI-6 Standard: Into a 10-mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with MMB-4, deliver 10 µL of the 10 mg/mL HI-6 stock prepared in Section G.3.d. (using a 10 µL gas-tight syringe). Mix well and dilute to volume with mobile phase diluent. Mix on a vortex mixer.

(5) Blank: The mobile phase diluent prepared in G.3.c. is used as the HI-6 blank.

g. MMB-4 Standards: (Must be made up fresh daily.)

(1) 100 µg/mL MMB-4 Standard: Into a 10 mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with HI-6 (G.3.e.), deliver 50 µL of the 20 mg/mL MMB-4 stock solution (using a 50 µl gas-tight syringe). Mix well and dilute to volume with mobile phase diluent.

(2) 50 µg/mL MMB-4 Standard: Into a 10-mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with HI-6, deliver 25 µL of the 20 mg/mL MMB-4 stock

prepared in Section G.3.d. (using a 25 µL gas-tight syringe). Mix well and dilute to volume with mobile phase diluent. Mix on a vortex mixer.

(3) 20 µg/mL MMB-4 Standard: Into a 10-mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with HI-6 prepared in Section F.2.e., deliver 10 µL of the 20 mg/mL MMB-4 stock prepared in Section F.2.d. (using a 10 µL gas-tight syringe). Mix well and dilute to volume with mobile phase diluent. Mix on a vortex mixer.

(4) 10 µg/mL MMB-4 Standard: Into a 10-mL volumetric flask containing approximately 5 mL of the mobile phase diluent spiked with HI-6, deliver 5 µL of the 20 mg/mL MMB-4 stock prepared in Section G.3.d. (using a 10 µL gas-tight syringe). Mix well and dilute to volume with mobile phase diluent. Mix on a vortex mixer.

(5) Blank: The mobile phase diluent prepared in F.2.e. is used as the MMB-4 blank.

3. Equipment Preparation:

a. Instrument Preparation: The HPLC is prepared for use with the following recommended initial settings and conditions. These settings and conditions are to be optimized by the operator prior to instrument calibration and sample analysis.

(1) Column - 15 cm x 4.6 mm inside diameter (I.D.) Zorbax LC-8 with 5 µm partial size or equivalent.

(2) Guard Column - 2 cm x 4.6 mm I.D. Zorbax LC-8 with 5 µm partial size.

(3) Mobile Phase: 80 percent buffer (see Section F.2.a)
15 percent Acetonitrile:Isopropanol (80:20)
5 percent spectroscopic grade methyl alcohol.

(4) Mobile Phase Flow Rate: 1.5 mL/min.

(5) Injection Volume: 100 µL volume for HI-6.
30 µL volume for MMB-4.

(6) Detector Integration Wavelength: 304 nm.

b. Column Conditioning: The column needs approximately 45 min of mobile phase conditioning before it can be used to analyze samples. This conditioning is required so the stationary phase can equilibrate with the ion-pair reagent in the mobile phase.

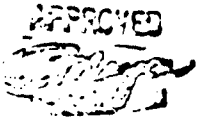
APPROVED

c. Column Check: The efficiency of the chromatographic system must be checked before samples are analyzed. To determine this a 25 µg/mL HI-6 standard is analyzed and the resolution and peak shape for each analyte peak, HI-6 and MMB-4 is determined. When reduction in column performance is observed, the column and or guard column must either be cleaned or replaced. The guard column should always be replaced and conditioned without the analytical column in position to avoid contamination of the analytical column.

4. Dilution of Samples: Samples are diluted in the mobile phase buffer. Samples having high concentrations of HI-6 or MMB-4 must be sonicated to insure complete solvation of the material, due to limited solubility of these materials in aqueous solvents, prior to performing dilutions. The expected concentration of each sample is adjusted to within the working calibration range. When sample size permits all samples will be diluted using volumetric glassware. When sample size is limited dilutions must be performed using analytical syringes. Before the samples are diluted, the volume of the stock solution prepared in either F.2.b. or F.2.d. must be calculated and added to the sample dilution scheme. The final concentration of the internal standard in the samples will be either 111 µg/mL for HI-6 or 25 µg/mL for MMB-4.

5. Instrument Calibration: Instrument calibration must be performed each time quantitation of samples is required. The instrument calibration is performed by injecting the analytical standards prepared in Section F.2.f. and F.2.g. A complete set of analytical standards is analyzed prior to analysis of any samples. Once the initial calibration and standards have been analyzed and the linearity confirmed, the sample may begin with at least every sixth sample injected being an analytical standard or blank to check the performance of the instrument. A complete set of analytical standards is analyzed following the last sample. All analytical standards analyzed are used to develop a complete calibration curve for quantitation of the samples. No sample amount may be reported that exceeds the range of the analytical standards. Samples that yield responses less than the calibration range will be reported as less than the lower quantitation limit. Any sample response that exceeds the largest analytical standard will be reported as greater than the highest analytical standard, and must be either diluted to within range or the calibration range extended for quantification of the sample.

6. Analysis of Analytical Blank: The appropriate analytical blank prepared in Section F.2.f.5.d. and F.2.g.5. is analyzed to determine the quality of the reagents used, and to determine if the presence of sample matrix components interfere with the analysis method.



7. Analysis of Samples: Samples and calibration standards are analyzed using the procedures described in Section F.5. At least every sixth analysis should be a analytical standard or blank.

8. Calculations: The sample amounts are calculated using an internal standard method of calibration. The calibration data is analyzed using a linear regression model to estimate the parameter values for the model.

   a. Using a simple linear regression program, enter the peak area ratio of HI-6 to MMB-4 as the ordinate (y-value) and the corresponding standard concentration as the abscissa (x-value). The regression model used to generate the slope, intercept, and correlation coefficient for HI-6 in the calibration data is:

$$y = bx + a$$

   b. If a regression program is not available, program the following calculations:

$$b = \frac{[(\Sigma y)(\Sigma x^2)-(\Sigma x)(\Sigma xy)]}{[n(\Sigma x^2)-(\Sigma x)^2]}$$

$$a = \frac{[n(\Sigma xy)-(\Sigma x)(\Sigma y)]}{[n(\Sigma x^2)-(\Sigma x)^2]}$$

$$r = \frac{[n(\Sigma xy)-(\Sigma x)(\Sigma y)]}{[(n(\Sigma x^2)-(\Sigma x)^2)^{\frac{1}{2}}(n\Sigma(y^2)-(\Sigma y)^2)^{\frac{1}{2}}]}$$

   where,

   y = ax + b
   a = slope
   b = y-intercept
   r = correlation coefficient
   x = analyte peak area/Internal standard peak area
   y = analyte amount in µg/mL
   n = number of replicates

   c. All data points obtained from the analysis of the analytical standards are used to calculate the regression model estimates. In addition the percent relative standard deviation (%RSD) between replicate standards should be monitored to insure that the instrument response does not change excessively during analysis.

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The %RSD for any set of analytical standards should not be greater than 10 percent. If the %RSD for any set is greater than 10 percent, the analysis is stopped and the problem corrected prior to resuming analyses. Do not include the results of the analytical blank analyses in the calibration calculations as this will weigh the regression toward zero.

d. Identify the analyte and internal standard peaks in the unknown sample chromatograms and record each peak area. The ratio of the analyte vs the internal standard is calculated and the value recorded. Using the regression values calculated from the calibration data, calculate the found concentration for each sample using the formula above.

9. Column Clean-Up: After each set of samples are analyzed, the column must be cleaned to remove proteins and peptides adhering to the stationary phase. This is accomplished by flushing the column with a series of solvents over a period of time. The sequence recommended for this clean-up is:

Millipore water at 1.5 mL/min for 60 min.

ACN at 1.5 mL/min for 10 min.

IPA at 1.0 mL/min 10 min.

50:50 IPA/chloroform ramped program
1.5 mL/min - 2.0 mL/min in 10 min
- 2.0 mL/min for 50 min

IPA at 1.5 mL/min for 5 min.

ACN at 1.5 mL/min for 10 min.

10. Instrument Shut-Down: When the instrument is not to be used for extended periods of time, the system must be shut down following manufacturer's instructions to ensure column life and instrument stability. The column clean-up procedure (Section G.10) is followed and the column is stored with 100 percent ACN wetting the stationary phase. The column must be tightly capped during storage to prevent solvent evaporation. The column must never be stored with buffer remaining on the support.

G. Emergency Procedures: All personnel involved in the HML or MREF laboratory operations, must be familiar with the respective laboratory's FSSP, and the emergency procedures detailed within this document. All personnel involved in the King Avenue operation must be familiar with HEG H/SP 8-01 and the emergency procedures detailed within this document.

H. First Aid Procedures: First aid and self aid at the MREF and hML are to be conducted as specified in the FSSP.

TLH:cah

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APPENDIX C

Pathology

TABLE 1. INDIVIDUAL ANIMAL GROSS PATHOLOGY DATA

Organ/Lesion	Animal Number	2	3	4	5	6	7	8	9	10	11	14	17	18	26	31
Lungs																
Congestion/Hemorrhage		1[a]	1	1	1	1	1	1	1	1	1	1	1	1		1
Trachea Mucosa																
Congestion/Hemorrhage		1		1		1										
Heart																
Congestion/Hemorrhage		1	1	1	1	1	1	1	1	1	1	1	1	1		1
Heart Pericardial Sac																
Fluid		1							1				1	1		
Heart AV Valve																
Hematocyst												1				
Thymus																
Congestion/Hemorrhage												1				
Small Intestine																
Congestion/Hemorrhage											1					
Mesentery																
Parasitic Cyst																
Liver																
Fibrosis, Parasitic																
Lymph Node Inguinal																
Abscess																

[a] A "1" indicates the presence of lesions in the associated organ.

TABLE 1. Continued

Organ/Lesion	Animal Number	32	34	35	36	40	42	45	46	47	48	49	50	51	52	53
Lungs																
Congestion/Hemorrhage		1	1	1	1	1		1	1	1	1	1	1	1	1	
Trachea Mucosa																
Congestion/Hemorrhage						1			1					1		
Heart																
Congestion/Hemorrhage			1	1	1	1		1	1	1	1	1	1	1	1	1
Heart Pericardial Sac																
Fluid			1	1										1	1	
Heart AV Valve																
Hematocyst																
Thymus																
Congestion/Hemorrhage								1		1		1				
Small Intestine																
Congestion/Hemorrhage		1				1		1								
Mesentery																
Parasitic Cyst							1									
Liver																
Fibrosis, Parasitic							1									
Lymph Node Inguinal																
Abscess																

TABLE 1. Continued

Organ/Lesion	Animal Number	55	56	88	90	96	98	99	101	102	103	106	107	108	109	110
Lungs																
Congestion/Hemorrhage		1	1	1		1		1	1	1	1	1	1	1	1	
Trachea Mucosa																
Congestion/Hemorrhage																
Heart																
Congestion/Hemorrhage		1	1	1			1	1	1	1	1	1	1	1	1	
Heart Pericardial Sac																
Fluid			1													
Heart AV Valve																
Hematocyst																
Thymus																
Congestion/Hemorrhage						1								1		
Small Intestine																
Congestion/Hemorrhage			1											1		
Mesentery																
Parasitic Cyst																
Liver																
Fibrosis, Parasitic																
Lymph Node Inguinal																
Abscess																

TABLE 1. Continued

Organ/Lesion	Animal Number	111	113	114	118	119	125	126	130	132	136	137	141	144	145	146
Lungs																
Congestion/Hemorrhage		1	1	1	1	1	1	1		1		1		1	1	
Trachea Mucosa																
Congestion/Hemorrhage																
Heart																
Congestion/Hemorrhage		1	1		1	1	1	1	1	1		1			1	
Heart Pericardial Sac																
Fluid			1													
Heart AV Valve																
Hematocyst																
Thymus																
Congestion/Hemorrhage						1	1		1	1	1					1
Small Intestine																
Congestion/Hemorrhage																
Mesentery																
Parasitic Cyst																
Liver																
Fibrosis, Parasitic																
Lymph Node Inguinal																
Abscess													1			

TABLE 2. SUMMARY OF GROSS PATHOLOGY

Organ/Lesion	Animals Examined: 60
Lungs	
Congestion/Hemorrhage	50
Trachea Mucosa	
Congestion/Hemorrhage	6
Heart	
Congestion/Hemorrhage	49
Heart Pericardial Sac	
Fluid	10
Heart AV Valve	
Hematocyst	1
Thymus	
Congestion/Hemorrhage	12
Small Intestine	
Congestion/Hemorrhage	6
Mesentery	
Parasitic Cyst	1
Liver	
Fibrosis, Parasitic	1
Lymph Node Inguinal	
Abscess	1

APPENDIX D

Pharmacokinetic Parameters

TABLE 1. RESULTS OF RIA ANALYSIS FOR ATROPINE CONCENTRATION (ng/mL)[a] FOLLOWING INJECTION WITH THREE WET/DRY AUTOINJECTORS

Animal Number	112	140	142	23	25	39	86	92	Mean	Standard Deviation
Body Weight (kg)	82.7	82.5	85.5	86.4	87.0	94.5	83.6	83.4	85.7	3.9
Time in Minutes After Injection										
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	11.8	0.0	25.6	9.3	1.4	10.2	24.3	17.3	12.5	9.5
2	21.4	1.5	26.4[b]	25.5	7.0	17.2	29.5	18.0	18.3	9.7
3	31.0	3.7	26.6[c]	27.4	9.6	19.6	27.5	19.8	20.7	9.6
4	21.5	3.7	26.5	21.0	11.4	17.8	26.2	17.1	18.2	7.6
5	20.3	4.6	23.9	21.4	9.6	16.8	24.4	17.4	17.3	6.9
6	20.1	6.8	23.8	18.9	11.7	16.2	25.4	17.1	17.5	6.1
8	17.6	6.7	24.4	13.3	12.8	15.8	23.8	17.5	16.5	5.8
12	17.4	9.9	23.4	11.4	13.5	14.5	25.3	17.2	16.6	5.5
16	23.8	11.4	23.6[d]	12.5	11.3	14.0	22.0	16.9	16.9	5.4
20	19.6[e]	11.8	21.5	12.0	12.0	11.7	18.1	16.4	15.4	4.0
40	14.8	11.0	14.2	13.2	11.1	10.5	11.6	12.0	12.3	1.6
60	10.0[f]	11.2	10.5	10.7	8.0	7.5	7.6	8.4	9.3	1.5
80	7.3	8.9	7.8	8.9	6.1	5.8[g]	6.1	7.0	7.2	1.2
120	4.8	6.3	5.3	6.3	3.8[h]	3.7	4.0	4.8	4.9	1.0
180	3.2	4.5	3.3[i]	4.5[j]	2.9	2.1	2.8[k]	3.0	3.3	0.8
240	2.4	3.2[l]	2.8	3.6[m]	2.2	2.1	2.3[n]	2.4	2.6	0.5
300	2.5	2.4[o]	2.4	3.0[p]	2.0	1.8[q]	2.2	2.0	2.3	0.4
360	1.7	1.8[r]	1.8[s]	1.6[t]	1.8	1.5	2.3[u]	1.5	1.8	0.3

[a]The minimum quantifiable concentration is 1 ng/mL.
[b]Actual time of blood sampling was 2.5 min.
[c]Actual time of blood sampling was 3.33 min.
[d]Actual time of blood sampling was 16.67 min.
[e]Actual time of blood sampling was 20.5 min.
[f]Actual time of blood sampling was 60.25 min.
[g]Actual time of blood sampling was 80.5 min.
[h]Actual time of blood sampling was 120.25 min.
[i]Actual time of blood sampling was 180.5 min.
[j]Actual time of blood sampling was 181.75 min.
[k]Actual time of blood sampling was 181 min.
[l]Actual time of blood sampling was 242.5 min.
[m]Actual time of blood sampling was 241.33 min.
[n]Actual time of blood sampling was 241 min.
[o]Actual time of blood sampling was 304 min.
[p]Actual time of blood sampling was 300.33 min.
[q]Actual time of blood sampling was 301.5 min.
[r]Actual time of blood sampling was 360.5 min.
[s]Actual time of blood sampling was 362 min.
[t]Actual time of blood sampling was 360.5 min.
[u]Actual time of blood sampling was 366 min.

TABLE 2. RESULTS OF RIA ANALYSIS FOR SERUM ATROPINE CONCENTRATION (ng/mL)[a] FOLLOWING INJECTION WITH THREE SYRINGES

Animal Number	112	140	142	23	25	39	86	92	Mean	Standard Deviation
Body Weight (kg)	82.7	82.5	85.5	86.4	87.0	94.5	83.6	83.4	85.7	3.9
Time in Minutes After Injection										
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	3.1	19.2	5.4	26.6	10.6	32.7	18.6	11.5	15.9	10.2
2	7.6	33.1	15.0	37.9	15.6	34.9[b]	20.5	23.6	23.5	10.9
3	11.1	29.6	18.2	36.5	16.3	[c]	17.7	25.0	22.0	8.8
4	14.3	27.9	19.4	30.1	15.5	25.1	18.5	21.3	20.5	5.7
5	12.8	25.1	23.8	29.7	15.2	24.1[d]	19.4	20.2	21.3	5.5
6	12.3	26.8	19.5	29.5	14.7	26.9	22.3	21.7	21.7	6.1
8	15.3	24.8	30.6	29.9	13.5	22.6	18.0	17.5[e]	21.5	6.5
12	13.3	23.6	31.3	29.0	14.4	25.2	24.0	23.1	23.0	6.3
16	12.8[f]	22.7	30.6	24.1	18.4	19.4	21.0	19.8[g]	21.1	5.1
20	17.2	17.8	25.8	28.2[h]	15.6	16.6	17.5	21.9	20.1	4.7
40	12.7	10.2	14.7	14.5	13.2[i]	10.8	10.4	16.9	12.9	2.4
60	10.5[j]	6.8	11.0	8.7[k]	10.7	8.0	7.0	10.0[l]	9.1	1.7
80	8.6	5.3	9.8	7.3[e]	8.5	5.0	4.7	7.4	7.1	1.9
120	5.4	3.9	6.3	4.7	5.5	4.0[m]	3.6	4.8	4.8	0.9
180	4.0	2.9[e]	6.3	3.1	3.8	2.9[n]	3.0	3.4	3.7	1.1
240	2.9	2.4[q]	2.5[r]	2.6	2.7	2.2	2.4	3.7	2.7	0.5
300	2.3	2.0	2.4[s]	2.2	2.2	2.1[t]	2.4	2.3	2.2	0.1
360	2.0	1.9	2.0	1.8	1.7	1.4[u]	1.9[v]	2.3[w]	1.9	0.3

[a] The minimum quantifiable concentration is 1 ng/mL.
[b] Actual time of blood sampling was 2.25 min.
[c] Blood sample was not obtained.
[d] Actual time of blood sampling was 5.5 min.
[e] Actual time of blood sampling was 8.5 min.
[f] Actual time of blood sampling was 16.25 min.
[g] Actual time of blood sampling was 16.33 min.
[h] Actual time of blood sampling was 20.25 min.
[i] Actual time of blood sampling was 40.5 min.
[j] Actual time of blood sampling was 60.5 min.
[k] Actual time of blood sampling was 62.75 min.
[l] Actual time of blood sampling was 60.25 min.
[m] Actual time of blood sampling was 80.5 min.
[n] Actual time of blood sampling was 120.33 min.
[o] Actual time of blood sampling was 181 min.
[p] Actual time of blood sampling was 182 min.
[q] Actual time of blood sampling was 241.5 min.
[r] Actual time of blood sampling was 243 min.
[s] Actual time of blood sampling was 302 min.
[t] Actual time of blood sampling was 301.5 min.
[u] Actual time of blood sampling was 364 min.
[v] Actual time of blood sampling was 361 min.
[w] Actual time of blood sampling was 361.33 min.

TABLE 3. PHARMACOKINETIC PARAMETERS FOR ATROPINE ESTIMATED FROM TWO-COMPARTMENT MODELS FOLLOWING INJECTION WITH THREE WET/DRY AUTOINJECTORS[a]

Animal Number Body Weight (kg)	23 86.4	25 87.0	39 94.5	86 83.6	92 83.4	112 82.7	140 82.5	142 85.5	Mean 85.7	Standard Deviation 3.9
C_{max} (ng/mL)	17.8	12.9	17.3	27.7	18.7	22.6	11.9	26.8	19.5	5.9
t_{max} (min)	6.9	12.9	4.7	2.7	2.4	4.6	29.7	2.0	8.2	9.4
$AUC_{0-\infty}$ (ng·min/mL)	2,398	2,106	2,316	2,773	2,132	2,489	2,103	2,679	2,374	260
K_e (min^{-1})	0.475	0.298	1.048	1.941	2.311	1.031	0.095	2.753	1.244	0.985
K_{el} (min^{-1})	0.020	0.007	0.008	0.011	0.009	0.010	0.007	0.010	0.010	0.004
V_{d_β} (L)	70	992	1,159	973	706	713	502	660	772	339
V_{d_β}/BW (L/kg)	0.81	11.40	12.26	11.64	8.46	8.62	6.08	7.72	8.37	3.74
A	274.50	11.96	15.64	26.00	14.93	20.17	-60.40	23.13		
B	13.29	3.51	3.36	3.76	4.44	4.24	75.85	4.69		
Alpha	0.413	0.015	0.022	0.027	0.016	0.018	0.005	0.019		
Beta	0.006	0.003	0.002	0.002	0.004	0.003	0.005	0.003		

[a]Atropine dose approximately 5.58 mg.

TABLE 4. PHARMACOKINETIC PARAMETERS FOR ATROPINE ESTIMATED FROM TWO-COMPARTMENT MODELS FOLLOWING INJECTION WITH THREE SYRINGES[a]

Animal Number Body Weight (kg)	23 86.4	25 87.0	39 94.5	86 83.6	92 83.4	112 82.7	140 82.5	142 85.5	Mean 85.7	Standard Deviation 3.9
C_{max} (ng/mL)	33.7	16.0	32.9	21.3	22.7	15.0	28.9	28.4	24.9	7.2
t_{max} (min)	3.0	4.5	0.0	3.1	5.0	11.8	3.8	17.6	6.1	5.7
$AUC_{0-\infty}$ (ng•min/mL)	2,782	2,223	2,287	2,252	2,768	2,371	2,503	2,890	2,510	268
K_a (min^{-1})	1.644	1.133	[b]	1.672	0.957	0.335	1.226	0.120	1.012	0.600
K_{el} (min^{-1})	0.013	0.008	0.014	0.010	0.009	0.007	0.013	0.021	0.012	0.005
V_{d_β} (L)	709	590	638	1,038	720	594	855	129	659	262
V_{d_β}/BW (L/kg)	8.20	6.78	6.75	12.42	8.63	7.18	10.37	1.50	7.73	3.18
A	32.03	15.12	26.51	19.58	20.24	11.70	28.37	143.30		
B	4.33	1.59	6.44	2.99	4.24	5.36	4.40	14.05		
Alpha	0.025	0.008	0.043	0.019	0.016	0.011	0.035	0.081		
Beta	0.003	0.004	0.004	0.002	0.003	0.004	0.003	0.006		

[a]Atropine dose approximately 5.58 mg.
[b]Meaningful value of K_a could not be obtained due to the extremely rapid absorption observed.

TABLE 5. MODEL-DERIVED AREAS UNDER THE SERUM ATROPINE CONCENTRATION-TIME CURVES TO EACH SAMPLING TIME FOLLOWING INJECTION WITH THREE WET/DRY AUTOINJECTORS

Animal	AUC_{0-1}	AUC_{0-2}	AUC_{0-3}	AUC_{0-4}	AUC_{0-6}	AUC_{0-8}	AUC_{0-12}	AUC_{0-16}	AUC_{0-20}
23	15.9	37.9	61.3	83.9	104.8	124.1	158.1	215.1	265.8
25	3.8	10.4	18.9	29.0	40.0	51.8	76.7	128.4	179.5
39	12.0	28.0	45.1	62.4	79.6	96.6	129.6	192.2	250.4
86	24.8	52.5	80.1	107.2	133.6	159.4	209.2	302.1	386.8
92	17.2	35.9	54.5	72.9	91.0	108.9	144.1	211.7	276.0
112	15.3	35.9	58.1	80.7	103.2	125.5	169.1	252.7	331.4
140	1.3	3.8	7.3	11.8	17.2	23.4	37.7	72.6	113.4
142	25.6	52.4	78.9	104.9	130.5	155.7	204.9	298.7	386.6
Mean	14.5	32.1	50.5	69.1	87.5	105.7	141.2	209.2	273.7
Standard Deviation	8.7	17.7	26.1	33.9	41.0	47.6	59.5	79.3	95.7

Animal	AUC_{0-30}	AUC_{0-40}	AUC_{0-60}	AUC_{0-80}	AUC_{0-120}	AUC_{0-180}	AUC_{0-240}	AUC_{0-300}	AUC_{0-360}
23	314.0	537.7	757.3	915.4	1,216.4	1,557.2	1,799.5	1,971.8	2,094.4
25	228.8	445.1	618.7	760.0	974.8	1,194.3	1,348.0	1,465.7	1,561.0
39	304.7	526.7	688.7	811.5	988.4	1,170.8	1,310.6	1,428.7	1,531.6
86	464.1	764.2	965.2	1,107.4	1,300.6	1,495.2	1,647.4	1,778.1	1,892.9
92	337.1	601.2	809.4	975.8	1,221.6	1,459.9	1,615.3	1,726.1	1,809.6
112	405.8	720.4	960.2	1,146.1	1,411.7	1,661.7	1,825.6	1,946.5	2,041.4
140	157.7	392.9	614.6	812.0	1,138.9	1,494.2	1,732.0	1,888.9	1,990.5
142	469.1	812.8	1,068.6	1,263.3	1,536.7	1,792.7	1,963.3	2,091.4	2,193.4
Mean	335.1	600.1	807.8	973.9	1,223.6	1,478.3	1,655.2	1,787.1	1,889.3
Standard Deviation	109.4	152.5	172.5	182.5	194.1	212.1	228.5	238.5	242.0

TABLE 6. MODEL-DERIVED AREAS UNDER THE SERUM ATROPINE CONCENTRATION-TIME CURVES TO EACH SAMPLING TIME FOLLOWING INJECTION WITH THREE SYRINGES

Animal	AUC_{0-1}	AUC_{0-2}	AUC_{0-3}	AUC_{0-4}	AUC_{0-5}	AUC_{0-6}	AUC_{0-8}	AUC_{0-12}	AUC_{0-16}
23	28.5	61.9	95.6	128.8	161.2	193.0	254.4	369.3	474.6
25	11.2	25.9	41.7	57.7	73.7	89.6	121.1	182.7	242.5
39	31.8	62.5	92.1	120.7	148.4	175.1	225.9	317.9	398.8
86	17.9	39.0	60.2	81.3	102.0	122.4	162.1	237.6	308.2
92	14.7	35.0	57.1	79.7	102.4	124.9	169.0	254.0	334.4
112	4.7	12.7	23.1	35.1	48.2	62.0	91.1	151.1	210.6
140	22.2	50.1	79.2	108.0	136.0	163.3	215.4	311.0	396.0
142	6.6	18.7	35.3	55.5	78.7	104.0	159.6	279.4	398.6
Mean	17.2	38.2	60.5	83.3	106.3	129.3	174.8	262.9	345.4
Standard Deviation	9.8	18.8	26.7	33.5	39.5	44.9	54.6	72.3	88.8

Animal	AUC_{0-20}	AUC_{0-40}	AUC_{0-60}	AUC_{0-80}	AUC_{0-120}	AUC_{0-180}	AUC_{0-240}	AUC_{0-300}	AUC_{0-360}
23	571.0	948.3	1,202.4	1,381.0	1,615.2	1,831.7	1,985.9	2,110.1	2,213.7
25	300.4	564.6	791.2	985.6	1,295.9	1,616.6	1,821.9	1,954.3	2,040.6
39	470.3	730.1	897.1	1,020.6	1,211.3	1,431.7	1,604.5	1,741.6	1,850.5
86	374.3	647.6	848.0	998.5	1,206.2	1,398.1	1,527.6	1,628.5	1,712.4
92	410.8	738.0	992.7	1,193.8	1,486.7	1,767.5	1,952.9	2,089.9	2,198.2
112	268.3	529.8	750.6	938.1	1,235.4	1,546.4	1,755.7	1,902.6	2,009.5
140	471.8	750.5	925.2	1,046.8	1,218.5	1,408.6	1,564.4	1,697.2	1,811.0
142	509.7	916.1	1,170.9	1,367.7	1,684.6	2,038.7	2,288.6	2,465.1	2,589.7
Mean	422.1	728.1	947.3	1,116.5	1,369.2	1,629.9	1,812.7	1,948.7	2,053.2
Standard Deviation	103.9	149.7	166.1	175.6	196.9	231.7	257.9	273.5	281.0

TABLE 7. RESULTS OF CHEMICAL ANALYSIS FOR PLASMA HI-6 CONCENTRATION (µg/mL)[a] FOLLOWING INJECTION WITH THREE MET/DRY AUTOINJECTORS

Animal Number Body Weight (kg)	140 82.5	92 83.4	112 82.7	86 83.6	25 87.0	142 85.5	23 86.4	39 94.5	Mean 85.7	Standard Deviation 3.9
Time in Minutes After Injection										
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	0.0	9.5	5.0	9.5	0.0	9.5	4.0	4.4	5.2	4.0
2	0.0	17.2	15.2	17.6	3.4	18.6[b]	13.7	8.6	11.8	7.0
3	0.0	21.4	21.9	22.1	6.7	22.2[c]	18.5	11.9	15.6	8.5
4	0.0	23.3	25.8	24.0	8.5	22.5	15.9	12.8	16.6	9.1
5	0.0	25.2	28.1	24.5	8.8	23.0	19.3	15.6	18.1	9.5
6	2.7	26.1	30.1	27.6	11.4	24.8	17.0	14.8	19.3	9.5
8	3.4	29.2	30.7	28.1	12.1	27.7	19.1	16.0	20.8	9.8
12	5.4	34.1	36.1	32.1	16.2	30.7	19.8	17.1	23.9	10.9
16	6.2	35.3	41.9	32.9	15.5	31.9[d]	[e]	18.9	26.1	12.7
20	7.0	35.4	42.0[f]	30.5	16.0	31.1	23.8	19.3	25.6	11.3
40	8.8	29.6	36.6	24.9	13.3	23.4	49.8	17.2	25.5	13.3
60	8.4	21.8	26.9[g]	18.0	10.5	16.5	23.3	13.3	17.3	6.5
80	7.1	16.0	19.7	12.5	6.9	14.9	18.6	9.1[h]	13.1	5.0
120	4.3	8.8	10.9	6.6	3.0[i]	8.3	9.7	5.4	7.1	2.8
180	0.0	3.3	3.7	2.7[j]	0.0	3.0[k]	5.5[l]	3.1	2.7	1.8
240	0.0[m]	0.0	0.0	0.0[n]	0.0	0.0	3.1[o]	0.0	0.4	1.1
300	0.0[p]	0.0	0.0	0.0	0.0	0.0	0.0[q]	0.0[r]	0.0	0.0
360	0.0[s]	0.0	0.0	0.0[t]	0.0	0.0[u]	0.0[v]	0.0	0.0	0.0

[a]The minimum quantifiable concentration is 2.5 µg/mL
[b]Actual time of blood sampling was 2.5 min.
[c]Actual time of blood sampling was 3.33
[d]Actual time of blood sampling was 16.67 min.
[e]Sample lost due to breakage of tube in centrifuge.
[f]Actual time of blood sampling was 20.5 min.
[g]Actual time of blood sampling was 60.25 min.
[h]Actual time of blood sampling was 80.5 min.
[i]Actual time of blood sampling was 120.25 min.
[j]Actual time of blood sampling was 181 min.
[k]Actual time of blood sampling was 180.5 min.
[l]Actual time of blood sampling was 181.75 min.
[m]Actual time of blood sampling was 242.5 min.
[n]Actual time of blood sampling was 241 min.
[o]Actual time of blood sampling was 241.33 min.
[p]Actual time of blood sampling was 304 min.
[q]Actual time of blood sampling was 300.33 min
[r]Actual time of blood sampling was 301.5 min.
[s]Actual time of blood sampling was 360.5 min.
[t]Actual time of blood sampling was 366 min.
[u]Actual time of blood sampling was 362 min.
[v]Actual time of blood sampling was 360.5 min.

TABLE 8. RESULTS OF CHEMICAL ANALYSIS FOR PLASMA III-6 CONCENTRATION (μg/mL)[a] FOLLOWING INJECTION WITH THREE SYRINGES

Animal Number	25	142	23	39	140	92	112	86	Mean	Standard Deviation
Body Weight (kg)	87.0	85.5	86.4	94.5	82.5	83.4	82.7	83.6	85.7	3.9
Time in Minutes After Injection										
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	0.0	2.7	17.1	16.2	8.6	3.1	2.6	8.7	7.4	6.5
2	10.1	9.3	36.9	30.4[b]	21.3	13.6	5.1	10.8	17.2	11.3
3	12.3	16.7	45.0	[c]	29.4	18.2	7.5	13.2	20.4	12.8
4	15.1	21.2	44.5	35.0	30.9	19.2	11.4	16.5	24.2	11.4
5	15.7	26.5	48.5	35.3[d]	33.9	15.4	12.2	19.5	25.9	12.6
6	15.1	27.4	49.8	39.5	37.0	20.9	14.4	22.7	28.3	12.6
8	16.5	33.8	49.2	41.2	39.9	22.2[e]	16.3	22.8	30.2	12.5
12	19.5	35.4	52.2	42.9	43.0	27.4	19.0	30.6	33.8	11.9
16	23.1	36.5	52.8	42.1	42.5	27.4[f]	20.3[g]	29.8	34.3	11.1
20	22.2	36.3	53.2[h]	39.8	51.0	28.5	23.6	29.7	35.5	11.8
40	23.3[i]	30.1	40.1	26.9	33.7	24.7	25.6	24.1	28.5	5.8
60	18.6	22.5	24.2[j]	19.8	23.5	19.0[k]	23.7[l]	17.8	21.1	2.6
80	13.3	17.2	18.3[m]	12.5	16.7	13.7	20.0	11.3	15.4	3.1
120	6.1	8.6	8.0	5.5[n]	7.5	7.1	12.5	6.4	7.7	2.2
180	0.0	3.5	3.1	2.8[o]	2.9[p]	2.8	7.1	3.1	3.2	1.9
240	0.0	0.0[q]	0.0	0.0	0.0[r]	0.0	4.1	0.0	0.5	1.4
300	0.0	0.0[s]	0.0	0.0[t]	0.0	0.0	2.4	0.0	0.3	0.8
350	0.0	0.0	0.0	0.0[u]	0.0	0.0[v]	0.0	0.0[w]	0.0	0.0

[a]The minimum quantifiable concentration is 2.5 μg/mL.
[b]Actual time of blood sampling was 2.25 min.
[c]Blood sample was not obtained.
[d]Actual time of blood sampling was 5.5 min.
[e]Actual time of blood sampling was 8.5 min.
[f]Actual time of blood sampling was 16.33 min.
[g]Actual time of blood sampling was 16.25 min.
[h]Actual time of blood sampling was 20.25 min.
[i]Actual time of blood sampling was 40.5 min.
[j]Actual time of blood sampling was 62.75 min.
[k]Actual time of blood sampling was 60.25 min.
[l]Actual time of blood sampling was 60.5 min.
[m]Actual time of blood sampling was 80.5 min.
[n]Actual time of blood sampling was 120.33 min.
[o]Actual time of blood sampling was 182 min.
[p]Actual time of blood sampling was 181 min.
[q]Actual time of blood sampling was 243 min.
[r]Actual time of blood sampling was 241.5 min.
[s]Actual time of blood sampling was 302 min.
[t]Actual time of blood sampling was 301.5 min.
[u]Actual time of blood sampling was 364 min.
[v]Actual time of blood sampling was 361.33 min.
[w]Actual time of blood sampling was [illegible]

TABLE 9. PHARMACOKINETIC PARAMETERS FOR HI-6 ESTIMATED FROM ONE-COMPARTMENT MODELS FOLLOWING INJECTION WITH THREE WET/DRY AUTOINJECTORS[a]

Sheep Number	25	142	23	39	140	92	112	86	Mean	Standard Deviation
D (mg/kg)	15.9	16.1	16.0	14.6	16.7	16.5	16.7	16.5	16.1	0.7
C_{max} (μg/mL)	16.4	31.1	36.7	19.1	8.9	35.3	41.7	32.2	27.7	11.5
C_{max}/D (kg/L)	1.0	1.9	2.3	1.3	0.5	2.1	2.5	2.0	1.7	0.7
t_{max} (min)	19.8	12.6	30.4	14.4	43.7	14.6	17.7	11.9	20.7	11.0
V_d (L)	60.14	37.62	21.14	62.06	65.36	32.33	26.15	36.36	42.65	17.34
K_e (min^{-1})	0.112	0.247	0.052	0.222	0.026	0.201	0.150	0.261	0.159	0.089
K_{el} (min^{-1})	0.017	0.013	0.019	0.011	0.020	0.013	0.013	0.014	0.015	0.003
t_{b_e} (min)	6.19	2.81	13.26	3.12	26.33	3.44	4.62	2.65	7.80	8.26
$t_{b_{el}}$ (min)	40.8	54.1	36.6	64.7	35.0	54.4	52.2	50.3	48.517	10.215
CL (mL/min/kg)	11.7	5.6	4.6	7.0	15.7	4.9	4.2	6.0	7.5	4.1
$AUC_{0-\infty}$ (μg·min/mL)	1,354	2,858	3,450	2,076	1,065	3,339	3,981	2,750	2,609	1,032
AUC_{0-360} (μg·min/mL)	1,350	2,828	3,444	2,030	1,062	3,302	3,944	2,730	2,586	1,023
$AUC_{0-\infty}/D$ (kg·min/L)	85	178	216	142	64	202	238	167	161	62

[a]HI-6 dose approximately 1,380 mg.

TABLE 10. PHARMACOKINETIC PARAMETERS FOR HI-6 ESTIMATED FROM ONE-COMPARTMENT MODELS FOLLOWING INJECTION WITH THREE SYRINGES[a]

Sheep Number	25	142	23	39	140	92	112	86	Mean	Standard Deviation
D (mg/kg)	15.9	16.1	16.0	14.6	16.7	16.5	16.7	16.5	16.1	0.7
C_{max} (μg/mL)	23.3	37.5	54.6	42.9	46.3	28.3	26.1	30.2	36.1	11.0
C_{max}/D (kg/L)	1.5	2.3	3.4	2.9	2.8	1.7	1.6	1.8	2.3	0.7
t_{max} (min)	18.7	17.3	9.0	9.4	13.8	16.4	30.8	17.0	16.5	6.8
V_d (L)	49.28	28.51	22.13	27.44	23.76	39.67	40.10	34.77	33.21	9.39
K_e (min^{-1})	0.158	0.149	0.371	0.335	0.197	0.173	0.080	0.146	0.201	0.100
K_{el} (min^{-1})	0.010	0.015	0.015	0.017	0.016	0.012	0.009	0.016	0.014	0.003
t_{k_e} (min)	4.39	4.64	1.87	2.07	3.52	4.01	8.66	4.75	4.24	2.10
$t_{k_{el}}$ (min)	69.4	47.4	46.3	41.0	42.2	55.9	77.2	43.5	51.86	13.57
CL (mL/min/kg)	5.7	4.9	3.8	4.9	4.7	5.9	4.4	6.6	5.1	0.9
$AUC_{0-\infty}$ (μg•min/mL)	2,809	3,299	4,174	2,971	3,534	2,796	3,838	2,492	3,239	578
AUC_{0-300} (μg•min/mL)	2,727	3,280	4,154	2,963	3,523	2,761	3,667	2,483	3,195	564
$AUC_{0-\infty}/D$ (kg•min/L)	177	205	261	203	212	169	230	151	201	35

[a]HI-6 dose approximately 1,380 mg.

TABLE II. MODEL-DERIVED AREAS UNDER THE PLASMA HI-6 CONCENTRATION-TIME CURVES TO EACH SAMPLING TIME FOLLOWING INJECTION WITH THREE WET/DRY AUTOINJECTORS

Animal	AUC_{0-1}	AUC_{0-2}	AUC_{0-3}	AUC_{0-4}	AUC_{0-5}	AUC_{0-8}	AUC_{0-9}	AUC_{0-12}	AUC_{0-15}
25	0.6	3.6	8.6	15.4	23.8	33.5	56.4	111.6	174.1
142	2.1	11.7	27.0	46.7	69.7	95.2	151.3	273.1	397.2
23	0.8	4.9	12.0	21.9	34.4	49.3	85.6	179.9	295.8
39	1.2	6.5	15.1	26.3	39.5	54.3	87.2	160.3	236.4
140	0.1	0.8	2.0	3.7	5.8	8.4	14.8	32.0	54.2
92	2.0	11.4	26.6	46.6	70.3	97.0	156.8	291.2	432.0
112	1.9	10.8	25.7	45.6	69.7	97.3	161.1	310.2	473.5
86	2.3	12.7	29.3	50.4	75.0	102.0	161.2	288.1	416.1
Mean	1.4	7.8	18.3	32.1	48.5	67.1	109.3	205.8	309.9
Standard Deviation	0.8	4.4	10.2	17.6	26.1	35.6	56.3	100.9	146.4

Animal	AUC_{0-30}	AUC_{0-60}	AUC_{0-90}	AUC_{0-90}	AUC_{0-120}	AUC_{0-180}	AUC_{0-240}	AUC_{0-300}	AUC_{0-360}
25	239.3	544.4	775.8	942.1	1,145.1	1,278.5	1,326.6	1,343.9	1,350.2
142	518.2	1,045.8	1,455.2	1,772.1	2,207.3	2,556.1	2,717.9	2,792.9	2,827.7
23	426.4	1,147.7	1,792.3	2,286.6	2,894.5	3,271.0	3,392.7	3,431.8	3,444.3
39	311.8	650.9	925.6	1,147.5	1,471.1	1,757.9	1,908.8	1,988.2	2,030.0
140	80.4	244.1	419.1	575.1	801.9	971.2	1,033.6	1,054.9	1,061.9
92	571.7	1,191.2	1,674.4	2,048.9	2,564.1	2,978.2	3,170.9	3,260.6	3,302.4
112	640.2	1,404.4	2,004.2	2,464.8	3,089.2	3,578.7	3,799.5	3,899.1	3,944.1
86	539.9	1,072.1	1,476.6	1,783.6	2,193.5	2,506.8	2,643.8	2,703.6	2,729.8
Mean	416.0	912.6	1,315.4	1,627.6	2,045.8	2,362.3	2,499.2	2,559.4	2,586.3
Standard Deviation	190.9	390.8	550.7	672.5	829.3	942.5	991.4	1,013.5	1,023.5

TABLE 12. MODEL-DERIVED AREAS UNDER THE PLASMA NI-6 CONCENTRATION-TIME CURVES TO EACH SAMPLING TIME FOLLOWING INJECTION WITH THREE SYRINGES

Animal	AUC_{0-1}	AUC_{0-2}	AUC_{0-3}	AUC_{0-4}	AUC_{0-5}	AUC_{0-6}	AUC_{0-8}	AUC_{0-12}	AUC_{0-16}
25	1.1	6.0	14.3	25.4	38.8	54.1	89.4	172.0	262.6
142	1.7	9.8	23.3	41.4	63.3	88.4	146.2	281.1	429.3
23	5.3	28.0	62.6	105.0	152.6	203.4	310.3	527.7	737.9
39	3.9	20.7	46.7	78.9	115.4	154.7	237.9	408.9	574.4
140	2.7	15.2	35.4	62.0	93.6	129.1	208.6	386.1	570.7
92	1.4	8.1	19.1	33.7	51.2	71.1	116.5	220.7	332.6
112	0.7	3.9	9.5	17.3	27.0	38.6	66.3	137.1	222.4
86	1.4	7.9	18.8	33.3	51.0	71.2	117.8	226.8	345.6
Mean	2.3	12.5	28.7	49.6	74.1	101.3	161.6	295.1	434.3
Standard Deviation	1.6	8.3	18.2	30.0	42.9	56.3	83.3	134.1	178.5

Animal	AUC_{0-20}	AUC_{0-30}	AUC_{0-48}	AUC_{0-72}	AUC_{0-120}	AUC_{0-168}	AUC_{0-240}	AUC_{0-360}	AUC_{0-504}
25	355.7	793.6	1,158.4	1,457.4	1,902.7	2,311.3	2,535.7	2,658.8	2,726.5
142	578.1	1,255.4	1,773.2	2,160.2	2,664.7	3,035.2	3,189.1	3,253.1	3,279.7
23	937.2	1,774.2	2,394.8	2,854.9	3,448.9	3,878.3	4,053.3	4,124.6	4,153.6
39	730.7	1,373.6	1,832.1	2,159.0	2,558.1	2,821.1	2,916.3	2,950.9	2,963.4
140	752.0	1,525.4	2,087.0	2,491.6	2,992.9	3,331.4	3,457.9	3,505.1	3,522.8
92	445.5	956.6	1,360.6	1,675.9	2,113.9	2,471.7	2,641.7	2,722.4	2,760.8
112	317.1	832.4	1,313.5	1,725.7	2,362.3	2,976.7	3,335.3	3,544.6	3,666.8
86	466.4	1,007.2	1,411.4	1,706.1	2,076.3	2,332.2	2,430.7	2,468.5	2,483.1
Mean	572.8	1,189.8	1,666.4	2,028.9	2,515.0	2,894.7	3,070.0	3,153.5	3,194.6
Standard Deviation	217.1	351.5	428.9	474.8	517.7	537.9	548.1	556.8	563.7

APPENDIX E

Sample 1 and 2 Compartment Model Computer Programs

ONE-COMPARTMENT PHARMACOKINETIC MODEL

```
        ELSE IF X=5 THEN DO; SUMY5=SUMY; END;
        ELSE IF X=6 THEN DO; SUMY6=SUMY; END;
        ELSE IF X=8 THEN DO; SUMY8=SUMY; END;
        ELSE IF X=12 THEN DO; SUMY12=SUMY; END;
        ELSE IF X=16 THEN DO; SUMY16=SUMY; END;
        ELSE IF X=20 THEN DO; SUMY20=SUMY; END;
        ELSE IF X=40 THEN DO; SUMY40=SUMY; END;
        ELSE IF X=60 THEN DO; SUMY60=SUMY; END;
        ELSE IF X=80 THEN DO; SUMY80=SUMY; END;
        ELSE IF X=120 THEN DO; SUMY120=SUMY; END;
        ELSE IF X=180 THEN DO; SUMY180=SUMY; END;
        ELSE IF X=240 THEN DO; SUMY240=SUMY; END;
        ELSE IF X=300 THEN DO; SUMY300=SUMY; END;
        ELSE IF X=360 THEN DO; SUMY360=SUMY; END;
      END;
      INTAUC=SUMY;
      CALCAUC=D/V/KE;
      TKA=LOG(2)/KA;
      TKE=LOG(2)/KE;
      TMAX=LOG(KA/KE)/(KA-KE);
      CMAX=D/V*KA/KD*(EXP(-KE*TMAX)-EXP(-KA*TMAX));
      CMAX_D=CMAX/D_MG_KG;
      CL=D/CALCAUC;
      AUC_D=CALCAUC/D_MG_KG;
      DROP _TYPE_ _NAME_ _ITER_ SUMY D V KD;
    PROC PRINT;
      TITLE1 'SINGLE-COMPARTMENT MODEL WITHOUT IV DOSE';
      TITLE2 'PARAMETERS FOR TASK 89-06 HI-6 ADMINISTERED BY WET/DRY
AUTOINJECTOR: ANIMAL 23';
      VAR _SSE_ --Y INTAUC--AUC_D;
    PROC PRINT;
      VAR SUMY1--SUMY360;


    OPTIONS LS=80;
    DATA TRUNC;
      SET REG.ALLDATA;
      IF CONC NE 0 AND ANIMAL=23;
    PROC MEANS NOPRINT DATA=TRUNC;
      VAR T;
      ID ANIMAL;
      OUTPUT OUT=MAX MAX=MAXT;
    DATA MAX2;
      SET MAX;
       _TYPE_='FINAL';
    PROC SORT; BY _TYPE_;
    PROC NLIN DATA=TRUNC CONVERGE=1E-2 MAXITER=100 METHOD=MARQUARDT
OUTEST=ESTIM;
      PARMS A=15.99
            B=6.01
            ALPHA=0.022
            BETA=0.003
            KA=0.90;
     AEXP=EXP(-ALPHA*T);
```

```
      OPTIONS LS=80;
      DATA TRUNC;
        SET WETDRY.ALLDATA;
        IF ANIMAL=23 AND CONC NE 0;
      PROC MEANS NOPRINT DATA=TRUNC;
        VAR TIME; ID ANIMAL;
        OUTPUT OUT=MAX MAX=MAXT;
      DATA MAX2;
        SET MAX;
         TYPE_='FINAL';
      PROC SORT; BY _TYPE_;
      PROC MLIN DATA=TRUNC CONVERGE=1E-6 METHOD=MARQUARDT OUTEST=ESTIM;
        PARMS KA=0.1221
              KE=0.0188
              V=360;
      *   _WEIGHT_=1/CONC;
        D=16000;
        T=TIME;
        EXPA=EXP(-KA*T);
        EXPE=EXP(-KE*T);
        KD=KA-KE;
        MODEL CONC=D/V*KA/KD*(EXPE-EXPA);
        DER.KA=D/(V*KD)*((1-KA/KD)*(EXPE-EXPA)+KA*T*EXPA);
        DER.KE=D*KA/(V*KD)*((EXPE-EXPA)/KD-T*EXPE);
        DER.V=-D*KA/KD*(EXPE-EXPA)/V/V;
        TITLE 'SINGLE-COMPARTMENT PHARMACOKINETICS MODEL WITHOUT IV DOSE';
        TITLE2 'TASK 89-06 PLASMA HI-6, ADMINISTERED BY WET/DRY AUTOINJECTOR -
ANIMAL 23';
        OUTPUT OUT=WETDRY.P23 P=CONCHAT L95M=LCL U95M=UCL;
      PROC SORT DATA=ESTIM;
        BY _TYPE_;
      PROC PRINT DATA=WETDRY.P23;
      DATA EST;
        SET ESTIM;
        IF _TYPE_='FINAL';
      DATA WETDRY.AN23;
        MERGE EST MAX2; BY _TYPE_;
        KD=KA-KE;
        D=16000;
        V_l_kg=V/1000;
        D_MG_KG=D/1000;
        DX=0.5;
        X=0;
        SUMY=0;
        DO UNTIL(X GE 360);
        * ALTERNATIVELY, THE ABOVE STATEMENT COULD READ DO UNTIL (X GE MAXT);
          Y=D/V*KA/KD*(EXP(-KE*X)-EXP(-KA*X))*DX;
          X=X+DX;
          SUMY=SUMY+Y;
          PART=Y/SUMY;
               IF X=1 THEN DO; SUMY1=SUMY; END;
          ELSE IF X=2 THEN DO; SUMY2=SUMY; END;
          ELSE IF X=3 THEN DO; SUMY3=SUMY; END;
          ELSE IF X=4 THEN DO; SUMY4=SUMY; END;
```

TWO-COMPARTMENT PHARMACOKINETIC MODEL

```
  BEXP=EXP(-BETA*T);
  KEXP=EXP(-KA*T);
  MODEL CONC=A*(AEXP-KEXP)+B*(BEXP-KEXP);
  DER.A=AEXP-KEXP;
  DER.B=BEXP-KEXP;
  DER.ALPHA=-A*T*AEXP;
  DER.BETA=-B*T*BEXP;
  DER.KA=(A+B)*T*KEXP;
  TITLE 'TWO-COMPARTMENT ATROPINE PHARMACOKINETICS MODEL';
  TITLE2 'TASK 89-06 WET/DRY TECHNIQUE - ANIMAL 23';
  OUTPUT OUT=REG.P23OUT P=CONCHAT L95M=LCL U95M=UCL;
PROC SORT DATA=ESTIM;
  BY _TYPE_;
PROC PRINT DATA=REG.P23OUT;
DATA EST;
  SET ESTIM;
  IF _TYPE_='FINAL';
DATA REG.AN23;
  MERGE EST MAX2; BY _TYPE_;
  D=5600000;
  DX=1;
  X=-1;
  SUMY=0;
  PART=1;
  DO UNTIL (X GE 360);
  * ALTERNATIVELY, THE ABOVE STATEMENT COULD READ DO UNTIL (X GE MAXT);
    X=X+DX;
    Y=A*(EXP(-ALPHA*X)-EXP(-KA*X))+B*(EXP(-BETA*X)-EXP(-KA*X));
    SUMY=SUMY+Y;
    PART=Y/SUMY;
         IF X=1 THEN DO; SUMY1=SUMY; END;
    ELSE IF X=2 THEN DO; SUMY2=SUMY; END;
    ELSE IF X=3 THEN DO; SUMY3=SUMY; END;
    ELSE IF X=4 THEN DO; SUMY4=SUMY; END;
    ELSE IF X=5 THEN DO; SUMY5=SUMY; END;
    ELSE IF X=6 THEN DO; SUMY6=SUMY; END;
    ELSE IF X=8 THEN DO; SUMY8=SUMY; END;
    ELSE IF X=12 THEN DO; SUMY12=SUMY; END;
    ELSE IF X=16 THEN DO; SUMY16=SUMY; END;
    ELSE IF X=20 THEN DO; SUMY20=SUMY; END;
    ELSE IF X=40 THEN DO; SUMY40=SUMY; END;
    ELSE IF X=60 THEN DO; SUMY60=SUMY; END;
    ELSE IF X=80 THEN DO; SUMY80=SUMY; END;
    ELSE IF X=120 THEN DO; SUMY120=SUMY; END;
    ELSE IF X=180 THEN DO; SUMY180=SUMY; END;
    ELSE IF X=240 THEN DO; SUMY240=SUMY; END;
    ELSE IF X=300 THEN DO; SUMY300=SUMY; END;
    ELSE IF X=360 THEN DO; SUMY360=SUMY; END;
  END;
  INTAUC=SUMY*DX;
  CALCAUC=A/ALPHA+B/BETA-(A+B)/KA;
  D1=A*(KA-ALPHA)+B*(KA-BETA);
  K21=((A*BETA*KA)+(B*ALPHA*KA)-(A+B)*ALPHA*BETA)/D1;
  KEL=ALPHA*BETA/K21;
```

```
      K12=ALPHA*BETA-K21-KEL;
      V1=D/(A+B)/1000;
      Vdbeta=V1*KEL/BETA;
      TBETA=LOG(2)/BETA;
      TMAX=1/(KA-KEL)*LOG(KA/KEL);

CMAX=A*(EXP(-ALPHA*TMAX)-EXP(-KA*TMAX))+B*(EXP(-BETA*TMAX)-EXP(-KA*TMAX));
      DROP _TYPE_ _NAME_ _ITER_ SUMY D1;
    PROC PRINT;
      TITLE1 'TASK 89-15: TWO-COMPARTMENT PK MODEL FOR WET/DRY ATROPINE
TECHNIQUE';
      TITLE2 'PARAMETERS FOR ANIMAL 23;
      VAR _SSE_--Y INTAUC--CMAX;
    PROC PRINT;
      VAR SUMY1--SUMY360;
```